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Evaluating Terminal Differentiation of Porcine Valvular Interstitial Cells In Vitro

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Evaluating Terminal Differentiation of Porcine Valvular Interstitial Cells *In Vitro*

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Abstract

According to statistics from the American Heart Association, valvular heart disease directly leads to about 20,000 deaths a year and contributes to an additional 50,000. While significant advancements have been made in the treatment options available for valvular heart disease, complications still occur. For this reason, the future of valvular heart disease treatment lies in understanding the physiology of the heart valve, and subsequently bioengineering a valve from one's own tissue to mimic native valve processes.

Valvular interstitial cells (VICs) are the major cell type populating the valve matrix. In the inactive fibroblast-like state, these cells are responsible for extracellular matrix deposition. Activated VICs display a myofibroblast morphology characterized by the expression of alpha smooth muscle actin and are responsible for valve maintenance and repair. The activation of VICs is hypothesized to be stimulated by mechanical tension, which, in the presence of TGF-β1 allows the complete differentiation of VICs from the inactive to the active form. However, little is known about the potential for reversal or dedifferentiation from the active to inactive state. The purpose of this study was to determine whether substrate stiffness, the mechanical tension hypothesized to initiate VIC activation, modulates alpha smooth muscle actin expression in the presence and absence of TGF-β1. To mimic conditions found *in vivo,* substrates were varied from physiologic to pathological stiffness levels.

Results showed that when freshly isolated VICs are cultured in the presence of serum, alpha smooth muscle actin expression increased on all substrate stiffnesses. In TGF-β-free medium, there was an apparent increase on all stiffness levels as well, but a statistical significance between groups could not be demonstrated. Immunoblots used to detect TGF-β1 showed that intracellular TGF-β1 was upregulated in VICs cultured in the presence of serum compared to those cultured in TGF-β-free medium. Taken together, these results suggest that freshly isolated VICs become activated, as indicated by increased expression of alpha smooth muscle actin, on all substrate levels in the presence of serum. It also appears as though unknown factors which are present in serum are required to stimulate significant autocrine production of TGF-β1.

To determine whether VICs which had transitioned to the myofibroblast phenotype had the ability to dedifferentiate, cells were cultured on polystyrene for a minimum of four days then replated on substrates of varying stiffness. Analysis of alpha smooth muscle actin expression showed that, in the presence of serum and when replated on all of substrates used, alpha smooth muscle actin expression decreased, suggesting that these cells indeed have the potential to

dedifferentiate. A change in cell morphology to a more rounded phenotype as well as the loss of visible stress fibers further supported this possibility.

These studies represent a unique approach to studying phenotypic differentiation of valvular interstitial cells. Using acrylamide substrates of varying stiffness, and growth factor free media, we have shown that by altering substrate stiffness, changes in alpha smooth muscle actin expression consistent with differentiation and dedifferentiation can be induced. This potential for dedifferentiation suggests that in engineering the next generation of bioartificial valves, it may be possible to use the patient's own cells to seed the manufactured scaffold. This would avoid complications associated with current treatments, including immune rejections.

Acknowledgments

I would like to start by thanking Jill Rulfs, my academic advisor, for supporting me through my entire academic career. She has been an irreplaceable part of the six years I have spent at Worcester Polytechnic Institute. I would also like to thank my committee members, Dave Adams and Kristen Billiar for their guidance throughout this project. Furthermore, I owe much thanks to Angie Throm, my partner in crime during this project. For taking care of all office quandaries that have occurred while I've been at WPI, I am also very grateful to Carol Butler and Eileen Dagostino. I would like to give thanks to the UMass Core Flow Cytometry, particularly Richard Konz, for their assistance in analyzing my flow data in addition to Blood Farm for providing me with an unlimited supply of pig hearts. And last but not least, my family and friends for their unforgettable support during the past six years.

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Introduction

Heart Valve Disease and Treatments

According to the 2005 American Heart Association Heart Disease and Stroke Statistics Report, valvular heart disease leads to 19,989 deaths while contributing to 42, 590 (Thom, Haase *et al*. 2006). While significant advancements have been made in the treatment options available for valvular heart disease, the future of its treatment lies in understanding the physiology of the heart valve, and subsequently bioengineering a valve from one's own tissue to mimic native valve processes.

Types of Heart Valve Disease

Valvular heart disease, characterized by the loss of unidirectional flow of blood in the heart, can affect any heart valve; however, based on the number of reported cases in 2005, the valves most commonly affected are the mitral and aortic valves (Thom, Haase *et al*. 2006). One possible reason may be that the mitral and aortic valves are located on the left or "pumping" side of the heart, which withstands the most pressure and load since the left side pumps blood throughout the entire body before it returns to the heart and then lungs for re-oxygenation.

Valvular heart disease is generally caused by either congenital birth defects or age related degeneration. Congenital valvular heart disease is characterized by heart valves that are improper in size, do not close properly, or are misshapen. This disease most commonly affects the aortic and pulmonary valves, though the reasons for this are still unknown. Acquired valvular heart disease affects more people each year than congenital valvular heart disease and involves changes within the valve as a result of aging or infection that influence the deterioration of normal valve function. The most common causes of acquired heart valve disease include infective endocarditis, rheumatic fever, myxomatous degeneration, myocardial infarction, coronary artery disease, and cardiomyopathy (Schoen 1999).

Valvular stenosis and valvular insufficiency are the two most frequent types of valvular heart disease affecting both the aortic and mitral valves (Figure 1). When a valve becomes stenotic, the valve opening becomes stiff and blood no longer flows continuously along the appropriate pathway (Figure 1, left panel). As the disease progresses and the valve becomes restricted due to the characteristic stiffening of the valve, pressure builds in the heart chamber. This results in a reduction of blood flow throughout the heart and ultimately the body.

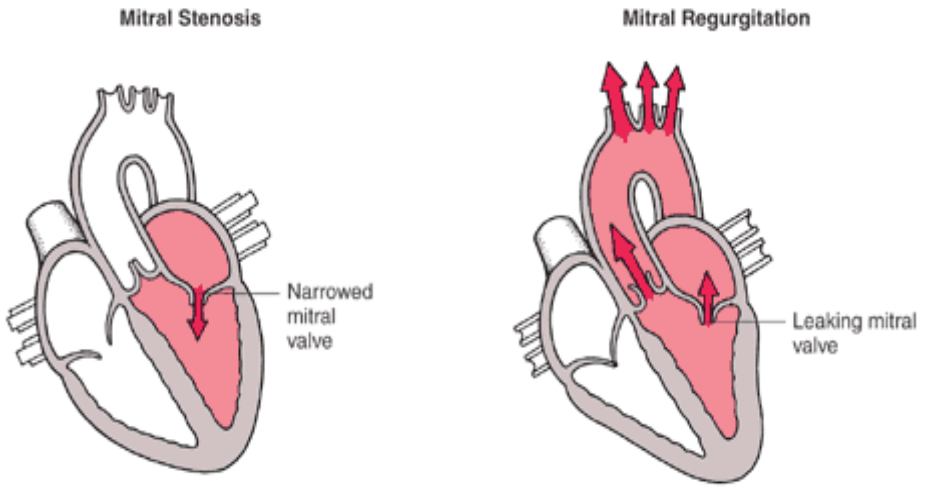

Figure 1: Characteristics of mitral stenosis and mitral regurgitation
Mitral stenosis (left panel) is characterized by a narrowing of the valve opening, which hinders continuous blood flow from one chamber to another. A second type of valvular heart disease is mitral regurgitation (right panel). This is caused by leaky or floppy valves that no longer close properly and decrease unidirectional flow of blood through the heart (Nishimura, Nishimura *et al*. 2002).

In contrast to stenosis, valves can also become loose or floppy (Figure 1, right panel). This form of valvular heart disease is identified as valvular insufficiency or regurgitation. Floppy valves no longer close effectively and blood begins leaking into the previous heart chamber. This leakage, whether small or large, results in a depressurization inside the heart and an improper pumping of blood throughout the body. Both types of valvular heart disease result in much more strain than is normally being put on the heart causing the walls of the heart to become enlarged. If not diagnosed and treated, this augmentation of the heart in combination with valvular heart disease can ultimately lead to other heart related complications.

Current Treatments and Associated Complications

There are several treatment options available for patients depending on the severity of the heart valve disease. Treatments range from valve repair for less severe cases to complete valve replacements. Valve replacement is performed using a prosthetic valve, while valve repair involves the restructuring of the patients own diseased valve.

Heart valve replacement research and development began as early as the 1950's with the focus on valve replacements with valves taken from fresh cadavers implanted into valvular heart disease patients (Schoen and Levy, 1999). Although the principal concepts and designs remain the same for all heart valve replacements, their design and production has improved significantly over the past 60 years through the enhancement of the valve replacement components.

The two types of heart valve replacements are mechanical and bio-prosthetics. Mechanical prosthetics are engineered heart valves made entirely from synthetic material. Mechanical prosthetics, though extremely durable with a potential lifespan of 25 years, present a high risk of thromboembolism due to clots forming on the synthetic valve surface (Schoen 1999; Schoen and Levy 1999). Because of the high risk of thrombosis, patients receiving mechanical valve replacements must take anti-coagulant drugs for the remainder of their lives.

Bio-prosthetic valve replacements are composed at least in part of human or animal tissue. The most common type of bio-prosthetic valve is the porcine valve, which is often used since the porcine heart is virtually identical in size to the human heart. In general, the lifespan of a bio-prosthetic valve ranges from 10 to 15 years depending on the age of the patient (Schoen and Levy 1999). Due to the limited lifetime of the replacement, a second and sometimes third surgery is required to replace valves that have failed due to age. Additionally, patients who receive bio-prosthetic valve replacements often have to take immunosuppressants to prevent an immune system response to the foreign tissue (Elkins, Dawson et al. 2001).

Based on the associated complications related to current valvular heart disease treatments, it is evident that further research in the development of bioengineered heart valves is necessary. Using a patient's own tissue for bioengineered valves would minimize the use of immunosuppressants as well as the need for anti-coagulant drugs therefore decreasing the number of associated complications correlated to valvular replacements. Further research on valve physiology is needed to comprehend the interactions of the cellular framework within the valves and its interactions with the surrounding environment.

Heart Valve Physiology

The human heart, a four-chambered organ, has four valves that work to maintain unidirectional flow and physiologic pressure within the heart (Figure 2). While all heart valves are required to maintain proper function within the heart, the aortic and mitral valve endure the most pressure at 80 mmHg during diastole and 120 mmHg during systole (Patton 2005). Anatomically, the aortic valve is located between the left atrium and aorta, and the mitral valve between the left atrium and ventricle.

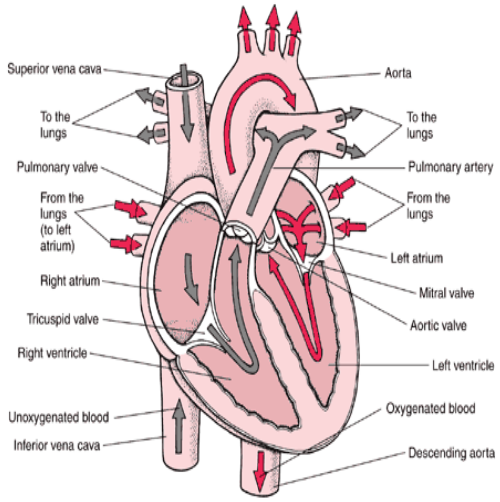

Figure 2: Anatomy of the heart and the normal direction of blood flow.
The heart contains four valves which normally maintain unidirectional flow within the heart. Blood flows from the lungs into the left atrium and ventricles then throughout the body. Blood then returns from the body to the right atrium and ventricles before reentering the lungs for re-oxygenation (**www.merck.com**).

A heart valve is composed of leaflets; the actual number of leaflets varies with the valve type. Each valve leaflet consists of three common layers: the fibrosa, spongiosa, and ventricularis (Figure 3). Each layer contains various extracellular matrix components, which act independently on valve function.

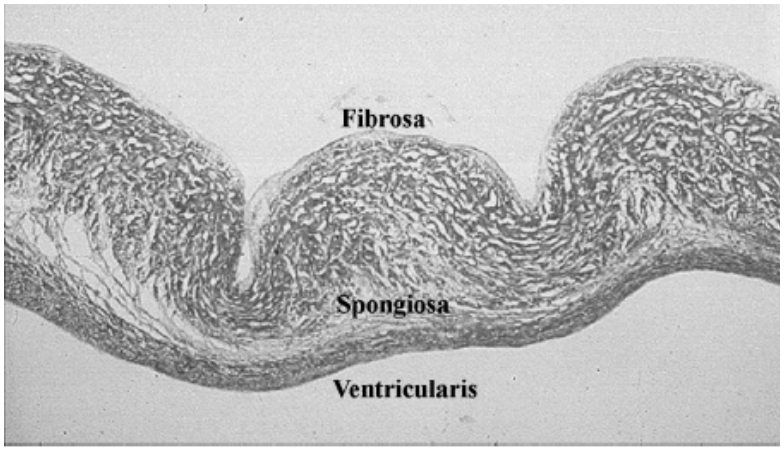

Figure 3: The three layers of a valve leaflet taken from the aortic valve.
The three layers of the valve, fibrosa, spongiosa, and ventricularis, make up each valve leaflet and work to maintain proper function of the valve through the various extracellular matrix components (Carew, Patel *et al*. 2003).

The fibrosa is located toward the outflow surface within the heart valve and is comprised primarily of collagen bundles. The fibrosa functions as the backbone providing strength and stiffness throughout the valve cusp. The spongiosa, located centrally within the valve cusp,

contains glucosaminoglycans (GAGs), which have a high affinity for water and therefore assist in hydrating the valve matrix while preventing damage caused by contraction of the valve. The final layer within the valve leaflet is the ventricularis, which is found on the inflow surface of the valve. The main extracellular matrix component of the ventricularis is elastin, which allows for repeated opening and closing of the valve while minimizing damage. Elastin also causes the leaflets to recoil to its diastole position after systole (Schoen 1999; Flanagan and Pandit 2003; Latif, Sarathchandra *et al*. 2005). A fourth layer is also found in atrioventricular valves such as the mitral valve. Similar to the ventricularis, this layer is also composed of elastin and functions in the recoil of the atrioventricular valves during closure (Flanagan and Pandit 2003).

Populating the extracellular matrix within the valve leaflets are two major cell types: valvular endocardial cells and valvular interstitial cells. Valvular endocardial cells form a continuous layer around the leaflet and although little is known regarding the function of these cells, Flanagan and Pandit (2003) suggest that the valvular endocardial cells may act to maintain a non-thrombogenic surface as well as regulate the underlying valvular interstitial cells.

Valvular interstitial cells (VICs) are the major cellular component of the heart valve and in the past decade have become the focus of numerous studies. Understanding how VICs behave both *in vivo* and *in vitro* will aid in understanding heart valve disease pathology in the design of bioengineered heart valve replacements for the treatment of valvular heart disease.

Valvular Interstitial Cell Biology

Valvular interstitial cells (VICs) are the most prevalent cell type found within heart valves. Researchers agree that morphologically, these cells tend to appear as fibroblasts with their polygonal shape and extended processes (Mulholland and Gotlieb 1996) (Figure 4).

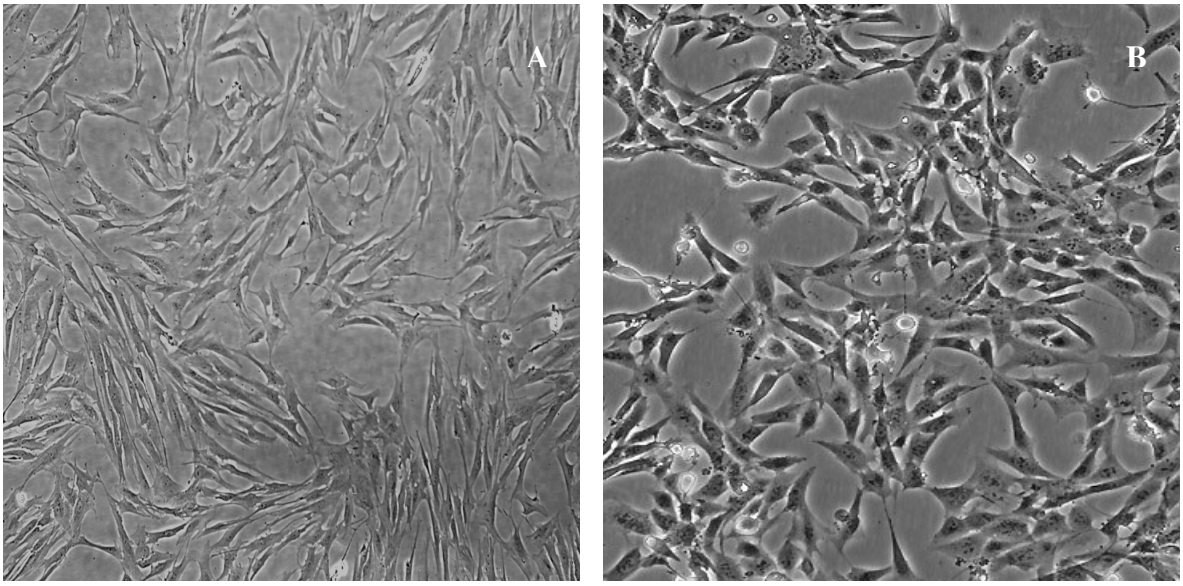

Figure 4: Valvular interstitial cells resemble fibroblasts *in vitro*
Valvular interstitial cells (A) resemble fibroblasts in culture (B) with their polygonal shape and extended processes. Valvular interstitial cells also express vimentin, the defining immunological marker of fibroblasts.

VICs have been classified as quiescent fibroblasts as the inactive phenotype and contractile myofibroblasts as the active phenotype (Mulholland and Gotlieb 1996; Gabbiani 2003; Rabkin-Aikawa, Farber *et al*. 2004). Inactive VICs, characterized as quiescent fibroblasts expressing the defining immunological marker vimentin, are the most common type of VIC found within a normal heart valve, at approximately 76.5% of total cells within the valve (Rabkin-Aikawa, Farber *et al*. 2004). The inactive VICs maintain valve function through the secretion of extracellular matrix. In addition to the large percentage of inactive VICs, a small percentage of VICs are characterized as contractile myofibroblasts and are required to repair damage caused by typical deterioration of the heart valve. These cells are identified through their expression of alpha smooth muscle actin, the marker for myofibroblasts. According to Rabkin-Aikawa and Farber *et al*. (2004) this activated VIC phenotype typically populates approximately 2.5% of the valve population (Mulholland and Gotlieb 1996; Bertipaglia, Ortolani et al. 2003; Rabkin-Aikawa, Farber et al. 2004).

Role of Valvular Interstitial Cells *In Vivo*

In vivo, inactive VICs characterized as quiescent fibroblasts work to maintain the extracellular environment, and have been recognized by the levels of rough endoplasmic reticulum and Golgi complexes present within the cell (Lester *et al*. 1988). These inactive VICs synthesize collagen, elastin, and proteoglycans, the key extracellular matrix components of the valve leaflet (Rabkin *et al.* 2001).

Though little is known regarding the mechanism of activation of the valvular interstitial cell *in vivo* from the quiescent fibroblast phenotype to the activated myofibroblast phenotype, it has been suggested by several researchers that this activation occurs through a change in the tissue microenvironment (MacKenna, Summerour et al. 2000). In a healthy heart, a mechanical stimulus is often the characteristic damage resulting from the stress sustained by the valves during systole and diastole. As stated previously, this continual need for valve repair results in a small percentage of the valvular interstitial cell population remaining in the activated phenotype in order to continually repair and maintain the valve (Lester, Rosenthal et al. 1988; Gabbiani 2003).

Implications of Stiffness on Valvular Interstitial Cell Activation *In Vitro*

In vivo, cells never experience the type of substrate represented by rigid glass or plastic surfaces typical of *in vitro* cell culture surfaces. In fact, it is believed that immediately upon culturing these cells under normal culture conditions, VICs undergo morphological and biochemical changes (Yperman 2004). As a replacement culture surface, Wang *et al*. (1998) have developed the use of polyacrylamide substrates for cell culture. Compared to collagen gels and other substrates developed for cell culture use, polyacrylamide substrates have mechanical properties that can more closely imitate environments found *in vivo*. These mechanical properties include the deformation of the substrate in proportion to a wide range of applied forces and the recovery of the substrate immediately upon releasing of the applied force. Coating the polyacrylamide substrate with extracellular matrix proteins such as fibronectin, provides direct interaction between the cell and the extracellular matrix proteins, further mimicking the cell's native environment (Wang and Pelham 1998).

By altering the percentage of bis-acrylamide and acrylamide, the stiffness of the polyacrylamide substrate can be manipulated to create both physiological and pathological environments *in vitro*. The typical range of stiffness for cell growth *in vivo* ranges from 0.01kPa to 10 kPa (Yeung, Georges *et al*. 2005). Using various cell types, researchers have identified several parameters that are affected by substrate stiffness even when the chemical parameters, such as media composition, remain constant. These parameters include the size, function, morphology and protein expression of various cell types (Yeung, Georges *et al*. 2005) (Figure 5).

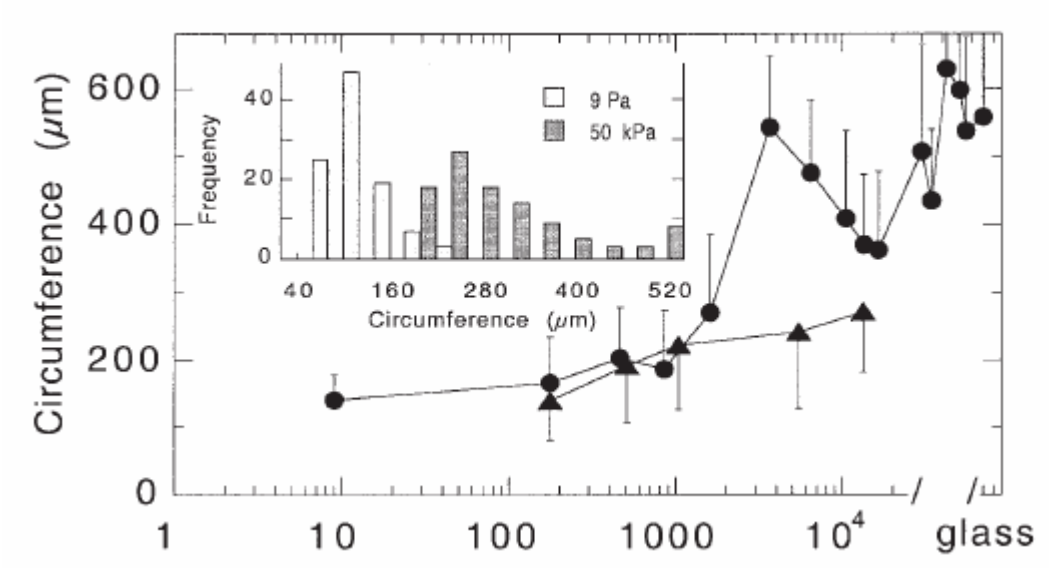

Figure 5 The effects of substrate stiffness on cell shape for 3T3 fibroblasts
3T3 fibroblasts were plated on substrates of varying stiffness coated with either fibronectin (circles) or collagen (triangles). Results showed that as substrate stiffness increased, the circumference of the fibroblasts in culture also increased. In addition to these results, it was also shown that the frequency of fibroblasts with different circumferences varied depending on substrate stiffness. (Yeung, Georges *et al*. 2005).

Engler *et al.* (2005) demonstrated that myotubes differentiate optimally on substrates of physiological stiffness levels. Myotubes plated on substrates with stiffness levels similar to those of native muscle tissue striated as they would *in vivo.* However, outside of this stiffness level, the myotubes striation never occurred. This result shows that cells respond to various stiffness levels by changes in morphology and phenotype and could have implications on the stem cell therapy when introduced to an altered mechanical environment than what is found normally.

Similarities Between Myofibroblast Differentiation and VIC Activation

While many aspects of VIC activation are still unknown, much is known about the differentiation of myofibroblasts during wound healing. The differentiation of myofibroblasts during wound healing has been hypothesized by several researchers to be a similar process to that of VIC activation as VICs are frequently characterized as myofibroblast-like cells(Mulholland and Gotlieb 1996; Bertipaglia, Ortolani et al. 2003; Flanagan and Pandit 2003).

In wounds, myofibroblasts have been defined as fibroblastic cells located within granulation tissue that exhibit a distinct cytoplasmic filamentous apparatus (Gabbiani 2003) (Figure 6). This cytoplasmic filamentous apparatus contains the defining immunological marker for myofibroblasts, alpha smooth muscle actin which is responsible for the contractile properties of myofibroblasts. Myofibroblast differentiation begins with the application of a mechanical

stimulus to the fibroblast cell type. *In vivo*, the probable cause would be injury to a tissue, while *in vitro* this may be a stiff culture environment or mechanical tension applied by a device. Once stimulated, the fibroblast is "activated" and develops into the proto-myofibroblast, the intermediary between the inactive fibroblast and the active, fully differentiated myofibroblast. The final step in the differentiation of myofibroblasts related to this thesis involves the combination of transforming growth factor beta 1 (TGF-β1) and mechanical tension (Vaughan, Howard *et al*. 2000). The source of TGF-β1 is platelets, macrophages, and parenchymal cells, which are localized at the wound site. Autocrine production by the fibroblasts is also a source of TGF-β1 and maintains the environment needed for the complete differentiation of myofibroblasts once the mechanical stimulus has disappeared and paracrine sources have dispersed (Gabbiani 2003).

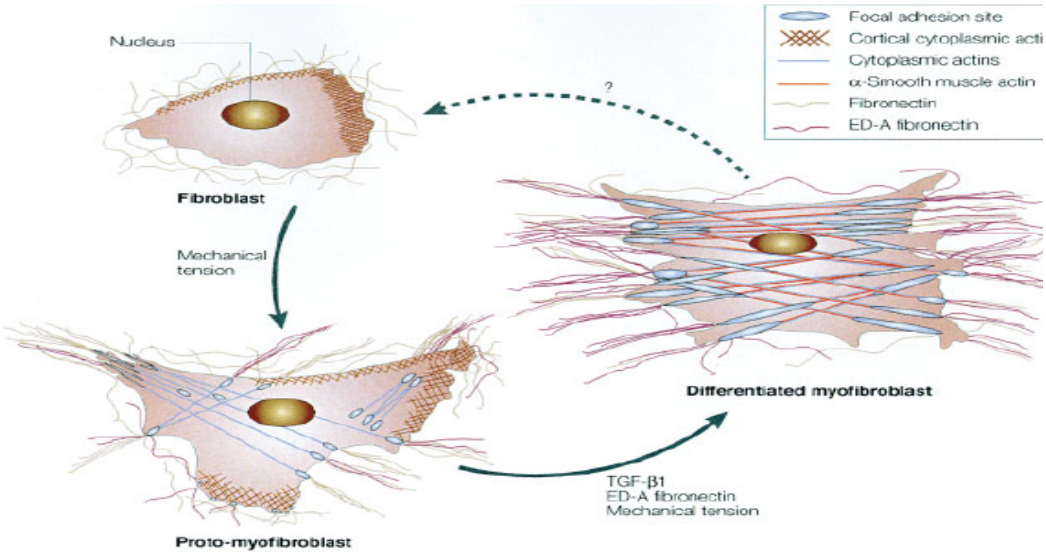

Figure 6 The two stages of myofibroblast differentiation
An inactive fibroblast (upper left) is stimulated by a form of mechanical tension and this begins the differentiation cycle. After stimulation, the activated fibroblast differentiates to the intermediary form, the proto-myofibroblast (lower left). The final step in myofibroblast differentiation involves the original mechanical stimulus, TGF-B1, and fibronectin. The combination of these three allows for the complete differentiation from the quiescent fibroblast to the activated, contractile myofibroblast (right side) (Tomasek, Gabbiani *et al*. 2002).

TGF-β1 stimulates alpha smooth muscle actin expression in fibroblasts both *in vivo* and *in vitro*. Subcutaneous administration of TGF-β1 in 6-week old Wistar rats showed an increase in granulation tissue, which is particularly abundant in alpha smooth muscle actin expressing myofibroblasts (Desmouliere, Geinoz *et al*. 1993). Cultured fibroblasts and myofibroblasts also respond to TGF-β1 addition with increasing expression of alpha smooth muscle actin (Tomasek, Gabbiani *et al*. 2002). The mechanical stimulus in each case is most likely the wound caused by

the syringe used to administer the dose of TGF- β1 and the stiff culture plastic used in most cell culture systems. In a study completed by Arora *et al. (1999)* fibroblasts were cultured in collagen gels from minimal to high compliance and then examined for the levels of alpha smooth muscle actin. Results showed that TGF-β1-induced increases of alpha smooth muscle actin are dependant on gel compliance. This further confirms that the combination of mechanical stimulus and TGF-β1 are required for myofibroblast differentiation.

Myofibroblast differentiation in the wound response is the closest available model for VIC differentiation from fibroblasts to myofibroblasts within the valve leaflet (Cushing, Liao *et al*. 2005). In normal wound response, myofibroblast contractility ceases with the deposit of collagen, re-epithelialization of the wounded tissue and high levels of differentiated myofibroblasts apoptosis (Tomasek, Gabbiani et al. 2002). On occasion, fibro-contractive diseases occur when this apoptosis never takes place and the highly contractile myofibroblasts continue to differentiate resulting in recurrent tightening of the tissue. This process may be analogous to the types of valvular heart diseases that result from a hyper-activation of the myofibroblast-like VIC phenotype, and lead to diseases such as myxomatous valve degeneration and valvular stenosis (Jian, Narula *et al.* 2002; Quick, Kunzelman *et al. 1997;* Rabkin-Aikawa, Farber *et al*. 2004).

Valvular Interstitial Cell and Disease

Valvular interstitial cell hyper-activation has been implicated in several types of heart valve disease from calcification of the valve to myxomatous degeneration(Schoen and Levy 1999; Rabkin, Aikawa et al. 2001; Jian, Narula et al. 2003). Similar to the pathology of fibrosis, valvular interstitial cells in a pathological state change the normal secretion of extracellular matrix proteins and enzymes, while maintaining their high contractility. This results in a valve that is unable to function properly. In a diseased state, the question remains whether these hyper-activated valvular interstitial cells can return to the quiescent fibroblast-like state, remain terminally differentiated as activated myofibroblasts or apoptose.

(Rabkin-Aikawa, Farber et al. 2004) assessed the phenotype of VICs under a variety of conditions using healthy, developing, injured, diseased, adapted and tissue engineered heart valves. Healthy valves showed the lowest number of activated VICs, though the actual percentage in a healthy valve is still controversial (Durbin and Gotlieb 2002). Myxomatous, short-term autografts and engineered valves showed the number of alpha actin-expressing cells to be 60.3% ± 9% of the total cell population. These cells also showed high levels of proteolytic activity. At 20 weeks post implantation for both adapted and tissue engineered valves, alpha

smooth muscle actin expression decreased significantly, demonstrating that cell populations in these two valves revert to a more normal distribution of myofibroblasts and fibroblasts, although whether this is a result of myofibroblast apoptosis or dedifferentiation is unclear. In the diseased group, diagnosed with myxomatous degeneration, no change in alpha smooth muscle actin expression was observed at later time points. This observation may be a result of the environment the cells are exposed to; myxomatous degeneration provides chronic pathological growth conditions, not suitable for the proper function of VICs *in vivo.*

Further studies completed by (Rabkin, Aikawa et al. 2001) demonstrate that activated VICs are involved in myxomatous degeneration, a disease characterized by floppy valves caused by inadequate extracellular matrix. Although activated VICs are not solely responsible for myxomatous degeneration, it is believed that they contribute to this disease through their role in extracellular matrix degradation and protease secretion within the heart valve (Lester *et al*. 1988). Results from this study showed that the majority of VICs within the myxomatous valves were in fact activated VICs expressing alpha smooth muscle actin.

While it appears clear that both the mechanical and biochemical environment regulate the differentiation status of valvular interstitial cells, the precise role of each in the differentiation pathway is not known. Since most of the studies were done in the presence of serum, separating out the roles of these two factors, stiffness and growth factor, is difficult. Likewise, it remains unclear whether VICs following activation and progression to the myofibroblast phenotype can revert to the non-contractile fibroblast phenotype and reduce their expression of alpha smooth muscle actin. Studies for this thesis are designed to elucidate the role of matrix stiffness and TGF-β1 in the differentiation of activated VICs and to determine whether alterations of these same factors can allow dedifferentiation to a non-contractile phenotype. Using polyacrylamide substrates of varying stiffness and growth factor free culture medium, we have investigated the role of each in regulating alpha smooth muscle actin expression.

The use of substrates whose stiffness can be varied allows observations of VIC phenotype on substrates of both physiological stiffness and pathological stiffness. This will allow for an improved comprehension of how the mechanical environment affects the activation of VICs. This will assist researchers in the design of scaffolds for the bioengineering of heart valve replacements in addition to further understanding how the pathology of heart valve disease is related to the hyper-activation of the myofibroblast-like valvular interstitial cell phenotype.

Materials and Methods

Cell Culture Media and Conditions

Control medium consisted of Dulbecco's Modified Eagles Medium (DMEM) (Cambrex) supplemented with 2mM L-glutamine, 10% heat inactivated fetal bovine serum, and 1X antibiotic/antimycotic solution (Sigma). TGF-β-free medium consisted of DMEM supplemented with 2mM L-glutamine, 2ng/ml basic fibroblast growth factor (bFGF), serum replacement 1 (Sigma) and 1X antibiotic/antimycotic solution (Sigma). All cells were cultured at 37^{o}C in 5% CO_2 and 95% air.

Mitral Valvular Interstitial Cell Isolation

Porcine hearts were obtained at Blood Farm (Groton, MA) and mitral valvular interstitial cells were isolated within 4 hours of slaughter. The mitral valve was excised and incubated at 37^{o}C for 30 minutes on a rocking platform in isolation medium consisting of 600U/ml collagenase (Worthington) and 1X antibiotic/antimycotic in DMEM. After initial digestion, single valve leaflets were rinsed in phosphate buffered saline (PBS), containing 10mM sodium phosphate pH 7.4, 150mM NaCl. The leaflets were then scraped on both sides to remove endocardial cells lining the leaflet surface and rinsed a second time in PBS. The leaflets were minced to approximately 0.5cm^2 pieces and placed into 3ml of fresh isolation medium and incubated at 37^{o}C for an additional 3 hours on a rocking platform. The cell suspension was then filtered through nylon mesh to remove any undigested pieces of tissue. The filtrate was split in to two tubes adding either 10ml control medium or TGF-β free medium and centrifuged at 1500 rpm for five minutes. The supernatant was aspirated and the cells were re-suspended in the appropriate medium. Cells were plated for 96 hours at a density of 250,000 cells per substrate.

Substrate Preparation

Glass slide activation, polyacrylamide preparation, and subsequent fibronectin treatment of the substrate surface were all completed according to the Wang *et al* (1998) protocol. The following modifications were made to this protocol: 51mm x 75mm glass slides were activated to promote adhesion of polyacrylamide to the glass surface (Electron Microscopy Science) and 45mm round cover slips were used to spread polyacrylamide on the glass slide surface. Polyacrylamide substrates were prepared by varying the ratio of acrylamide and bis-acrylamide to a desired stiffness of 7kPa and 75kPa. The final ratio of acrylamide to bis-acrylamide for the 7kPa substrate was 5%/0.025% and for the 75kPa substrate, 8%/0.8%(Engler, Griffin et al.

2004). To achieve a desired thickness of 75μm, 84μl of the polyacrylamide solution was placed on the activated glass slide and covered with a 45mm #1 round cover slip. Following polymerization of the polyacrylamide, substrates were rinsed in 50mM HEPES buffer pH 8.5, and the #1 cover slip was removed to expose the polyacrylamide surface. Fibronectin coating of the polyacrylamide substrate was then completed according to the Wang *et al* (1998) protocol. Substrates were UV sterilized for 15 minutes in a sterile hood and placed in a sterile 150mm culture dish (Corning). The substrates were equilibrated overnight at 37°C in 20ml of the appropriate culture media and checked for contamination. Glass slides were used as a control in all experiments and were also UV sterilized and equilibrated overnight at 37°C in the proper media.

Immunocytochemistry

Mitral valvular interstitial cells (VICs) grown on substrates and glass slides were fixed and permeabilized in 1X fixation and permeabilization buffer (BD Biosciences) for 20 minutes at 4°C. Substrates were rinsed in PBS and either stored in 1X permeabilization and wash (perm/wash) buffer (BD Biosciences) or stained for alpha smooth muscle actin using a monoclonal antibody. FITC conjugated anti-alpha smooth muscle actin (Sigma) was diluted 1:1000 in perm/wash buffer and incubated for one hour at 4°C. Substrates were washed overnight in 0.1% tween-20 in PBS to reduce background fluorescent due to residual antibody remaining within the polyacrylamide surface. The cells were then stained with a nuclear stain, Hoescht 33342 (Molecular Probes), at a dilution of 1:2000 in reagent grade de-ionized H_20 for 5 minutes at room temperature. After the nuclear stain, the substrates were washed three times in 0.1% tween-20 in PBS and then prepared for imaging.

Flow Cytometry

Cells were removed from glass or substrate through trypsinization and $1x10^6$ cells per experimental group were gathered for staining and processing. Cells were fixed and permeabilized using the same method as in immunocytochemistry. Cells were stained with a monoclonal antibody to alpha smooth muscle actin at 4°C for one hour using a dilution of 1:1000 in perm/wash buffer. Cells were rinsed three times in perm/wash buffer with centrifugation in between each wash step. AlexaFluor 700 (Molecular Probes) was diluted 1:2000 in perm/wash buffer and cells were incubated for 30 minutes at 4°C and then rinsed an additional three times in perm/wash buffer with centrifugation in between each wash step. Cells were re-suspended in 400μl of perm/wash buffer and analyzed using the LSR II flow cytometer (BD Biosciences).

Immunoblotting

Samples on all immunoblots were normalized to cell number. Pre-cast 10% tris-glycine polyacrylamide gels (Life Gels) were loaded with 50µl of prepared sample and run at 100 volts for approximately one hour. Protein was transferred to an Immobilon PVDF membrane (Millipore) using a semi-dry transfer apparatus and the immunoblot was run for 45 minutes at 250mA. The membrane was blocked for one hour using 0.2% non-fat dry milk in PBS and incubated overnight with monoclonal antibodies to alpha smooth muscle actin (Sigma) diluted at 1:400 or TGF-β1 diluted 1:250 both in PBS. Membrane was rinsed three times in PBS and then incubated for one hour with alkaline phosphatase conjugated goat anti-mouse IgG. Membrane was rinsed three additional times in PBS prior to detection with the Immunstar Chemiluminescence Goat Anti-Mouse Detection Kit (Biorad) and all images were taken using the Fluor-S Imager (Biorad).

Results

Comparing Alpha Smooth Muscle Actin Levels at Initial Isolation and 12 Hours Post-Isolation

In the initial isolation protocol, VICs were plated for 12 hours post-isolation to promote the isolation of a pure population of VICs assuming that fibroblast-like cells plate more rapidly than other cell types found in the cell suspension. Upon further investigation, no detectable α-smooth muscle actin expression was observed between VICs plated for 12 hours and VICs analyzed at the end of the 96-hour time frame. Based on this observation, flow cytometry with an α-smooth muscle actin monoclonal antibody was used to compare fresh isolates and VICs plated for 12 hours post-isolation in both TGF-β positive and negative media types. Figure 7 shows a considerable difference between freshly isolated VICs and VICs plated for 12 hours in control medium (containing TGF-β), and TGF-β free media with the percentage of cells expressing alpha smooth actin determined to be 40.44%, 82.06% and, 77.78% respectively.

Time Course to Determine Maximum Alpha Smooth Muscle Actin Expression

To determine the amount of time needed for maximum expression of alpha smooth muscle actin, an immunofluorescence time course was performed over four days of culturing freshly isolated valvular interstitial cells on glass in the presence of serum (Figure 8). Culturing VICs on glass in the presence of serum is known to induce alpha smooth muscle actin expression, indicating VIC activation to a myofibroblast phenotype(Lester, Rosenthal et al. 1988). At the initial isolation, all observed cells were negative for alpha smooth muscle actin. Twenty-four hours post-isolation, alpha smooth muscle actin expression was found to be perinuclear and at forty-eight hours, the bulk of alpha smooth muscle actin expression was dispersed throughout the cytoskeleton with no appreciable change in expression levels observed at times longer than forty-eight hours. Because of slower growth in serum free medium, experiments were run for 96 hours to ensure that VICs grown in growth factor free media would achieve comparable growth and expression levels.

The Differentiation of Valvular Interstitial Cells In Vitro

Alpha smooth muscle actin expression was used as an indicator of valvular interstitial cells differentiation to a myofibroblast phenotype *in vitro.* To determine the role of TGF-β1 in

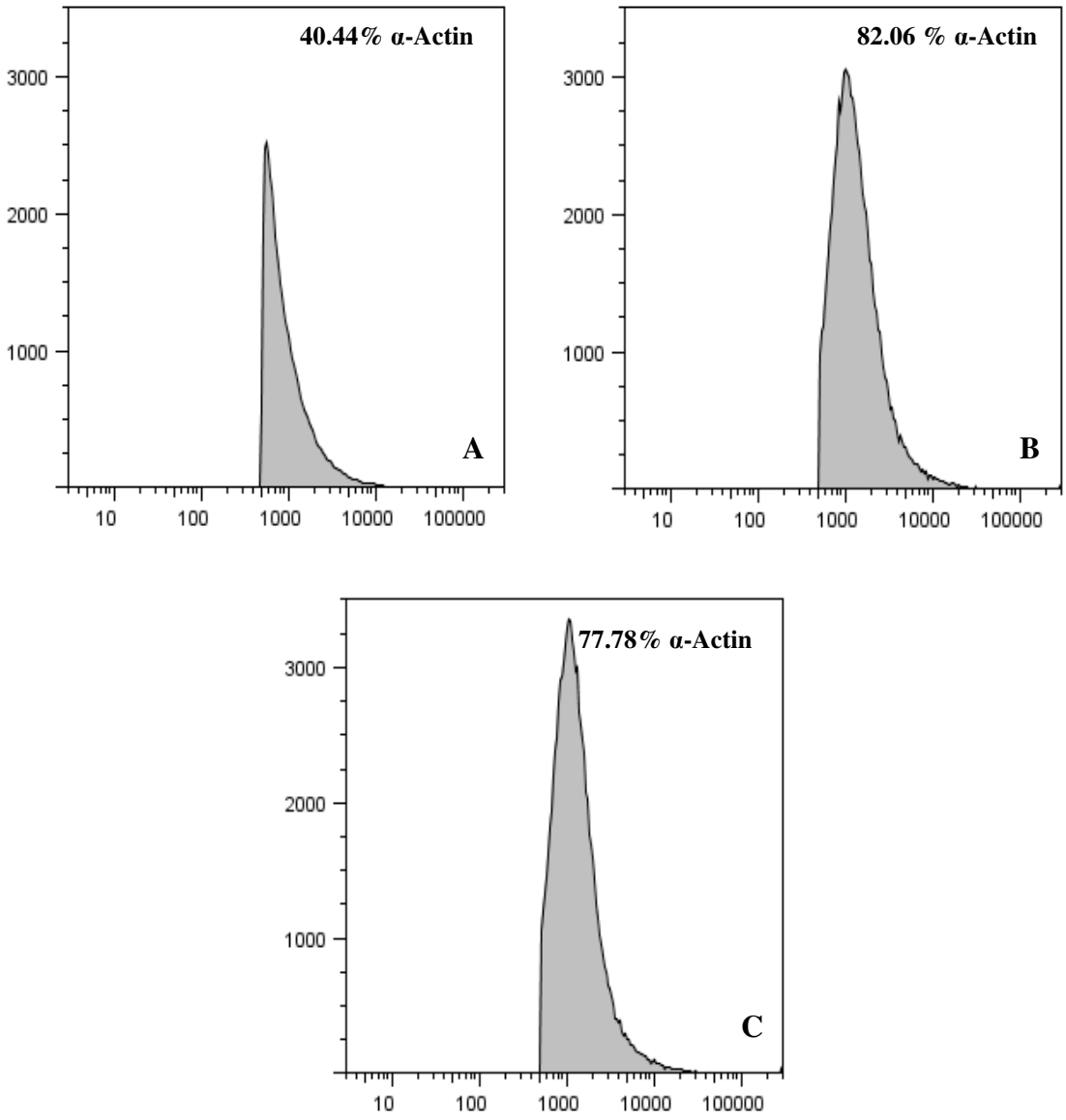

Figure 7: Alpha smooth muscle actin expression in VICs after initial and 12h post-isolation
Alpha smooth muscle actin expression in VIC populations after initial isolation (A), 12 hours post-isolation in control (B) and TGF-β free medium (C) as determined by FACS analysis. Cells were fixed as in Materials and Methods and labeled using a FITC-conjugated monoclonal anti-alpha smooth muscle actin antibody.

regulating alpha smooth muscle actin expression, cells were plated on the three substrates in the presence of 10% fetal bovine serum and in growth factor free medium. VICs were analyzed for alpha smooth muscle actin using flow cytometry at initial isolation and 96 hours post-isolation on the three varying substrate stiffnesses: 7kPa, 75kPa, Glass. Histogram plots from flow cytometry analyses were used to determine the percentage of VICs expressing alpha smooth muscle actin expression. Figure 9 shows representative FACS results comparing fresh isolates and cells grown on glass (maximum stiffness tested) in control medium for 96 hours, while Figure 10 shows a comparison of all three stiffness levels. While the absolute numbers from histogram plots varied from preparation to preparation, the trends for each data set were similar. These results from flow cytometry analyses of alpha smooth muscle actin illustrated that VICs cultured in control medium containing serum showed increased amounts of alpha smooth muscle actin expression on all three substrate stiffnesses in comparison to fresh isolates (Figure 10). In contrast, VICs cultured in TGF-β1 free medium (Figure 11) exhibited no statistical difference in alpha smooth muscle actin expression between substrates and of the fresh isolates. These results were further verified by immunocytochemistry using an antibody against alpha smooth muscle actin.

Immunocytochemistry comparing the two medium groups (Figure 12 and 13) demonstrated that alpha actin expression increased in VICs cultured in control medium (Figure 12) with a minimal increase in expression levels on the 7kPa and 75kPa substrate and a significant increase in expression in VICs plated on the glass substrate (Figure 12). VICs cultured in TGF-β1-free medium (Figure 13) showed no discernable alpha smooth muscle actin expression on the 7kPa and 75kPa substrates and low levels of alpha smooth muscle actin in VICs cultured on glass as compared to controls (Figure 12).

While these results appear quantitatively different from the flow cytometry results, this is likely due to a difference in the sensitivity of the technique. For both flow cytometry and immunocytochemistry, the results suggest the alpha smooth muscle actin expression increases with increasing substrate stiffness in serum containing medium. In TGF-β-free medium, the overall expression of alpha smooth muscle actin is significantly reduced at all levels of substrate stiffness and we were unable to demonstrate any statistically significant increase, although the data do indicate that there is some expression on glass substrates in the absence of TGF-β.

The Dedifferentiation of Valvular Interstitial Cells In Vitro

To determine whether this differentiation could be reversed, alpha smooth muscle actin expression was induced in VICs by culturing them in the appropriate medium on tissue culture

plastic for a minimum of four days. This resulted in a fully differentiated VIC phenotype indicated by high levels of alpha smooth muscle actin. Dedifferentiation was evaluated using a decrease in alpha smooth muscle actin expression by flow cytometry and immunocytochemistry (Figures 14-17).

Flow cytometry results using fully differentiation VICs cultured in control medium (Figure 14) as well as TGF-β1 free medium (Figure 15) showed no statistical decrease in alpha actin expression for cells plated on substrates of the varying stiffnesses: 7kPa, 75kPa, and 3gPa. For cells cultured in control medium containing FBS, no statistically significant differences could be demonstrated, but the mean values appeared to decrease with decreasing substrate stiffness. There may also be a decrease in cells cultured in TGF-β free medium, but it appears less apparent and not statistically demonstratable.

Immunocytochemistry results also suggested that alpha actin expression was reduced with decreasing substrate stiffness. VICs cultured in TGF-β-free medium (Figure 17) on both 7kPa and 75kPa substrates showed little to no expression in comparison to alpha actin expression in fully differentiated VICs (Figure 17). VICs plated on glass substrates expressed equivalent amounts of alpha smooth muscle actin as the control group of fully differentiated VICs. Minimal expression was also observed in VICs cultured in control medium (Figure 16) on substrates of 7kPa and 75kPa stiffness and as in TGF-B1 free cultured VICs, alpha actin expression was equivalent to levels found in alpha actin induced VICs (Figure 17). While these data are not quantifiable, they also indicate a change in alpha smooth muscle actin expression as a result of decreased substrate stiffness in both types of media. Cell morphology results from VICs cultured in the presence of serum (Figure 18) also illustrated a change in morphology between the stiff substrates, glass and 75kPa, and the 7kPa soft substrate reported to be in the range of physiologic stiffness levels found *in vivo*. Compared to VICs plated on glass, which were found to be polygonal in shape similar to those cultured on tissue culture plastic, VICs plated on the 7kPa substrate became much more rounded, a possible indication of dedifferentiation *in vitro.*

The Role of TGF-β1 in Valvular Interstitial Activation In Vitro

Because TGF-β1 is believed to play a significant role in VIC activation, immunoblots were used to assess autocrine production of TGF-β1 in cells cultured on substrates of varying stiffness (Figure 19). TGF-β1 levels were measured in fresh isolates and in VICs cultured for 96 hours in both media types on two varying substrate stiffnesses: 7kPa and 75kPa. In the presence of serum, TGF-β1 levels were significantly higher than those of fresh isolates or VICs cultured

in TGF-β1- free media, although some expression was evident in all samples (Figure 19). In dedifferentiation experiments using VICs cultured in TGF-β1 free medium or serum containing medium for 96 hours on substrates with stiffness values of 7kpa and 75kpa, no TGF-β1 was detectable.

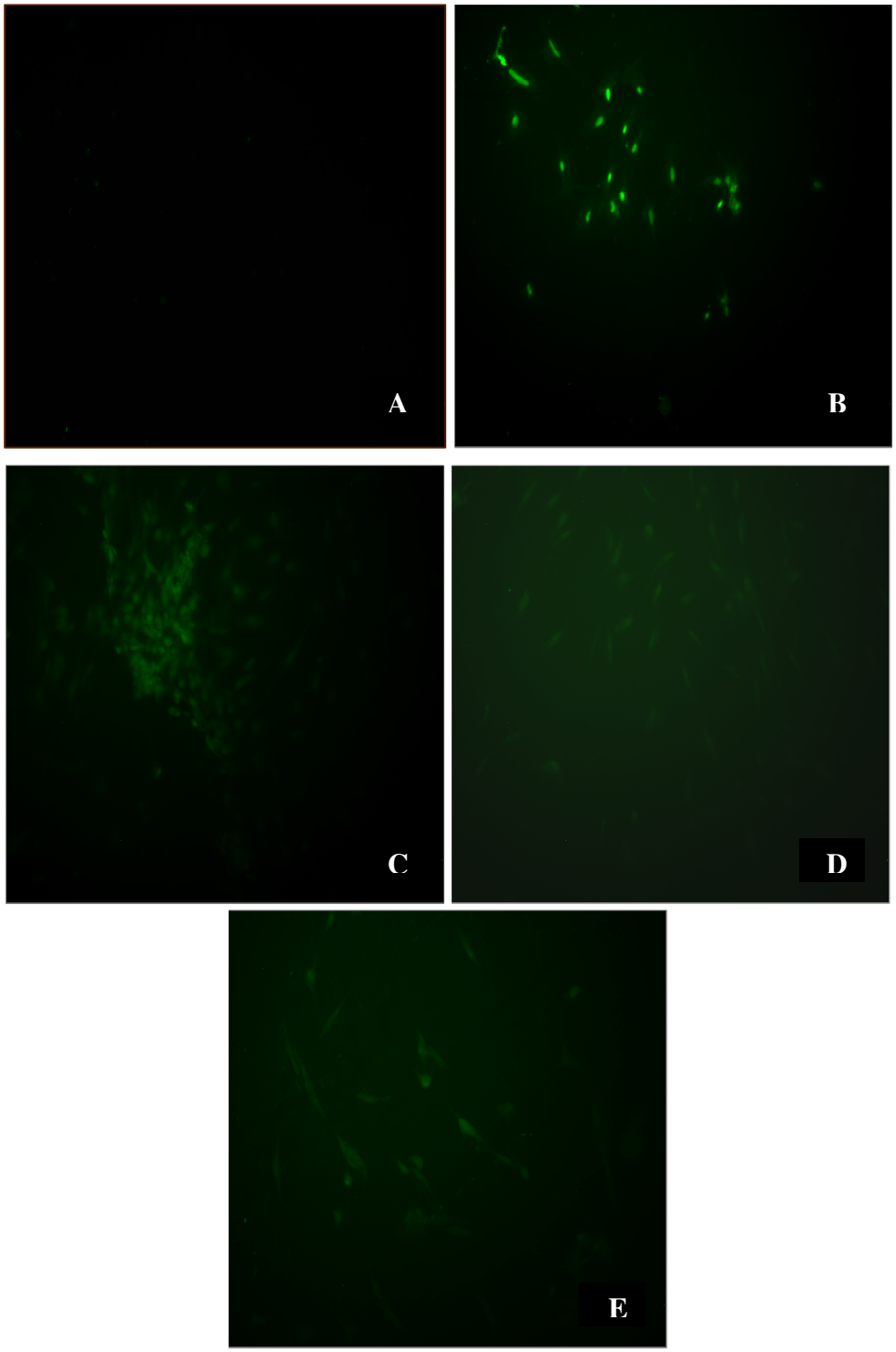

Figure 8: A 96-hour time course of VIC alpha actin expression.
VIC alpha smooth muscle actin expression over the course of 96 hours post-isolation. VICs were plated on glass and grown in control medium, two culture conditions known to induce the expression of alpha smooth muscle actin. Immunohistochemical staining for alpha smooth muscle actin was as described in Materials and Methods. No alpha smooth muscle actin expression was observed in fresh isolates (A). However, 24 hours post-isolation, alpha smooth muscle actin expression significantly increased and appeared to be mainly perinuclear (B). Maximum alpha smooth muscle expression was observed at 48 hours post isolation by which time staining was consistent with cytoskeletal distribution (C) and there were no significant changes in expression levels detected at 72 hour (D) and 96 hour (E) post-isolation.

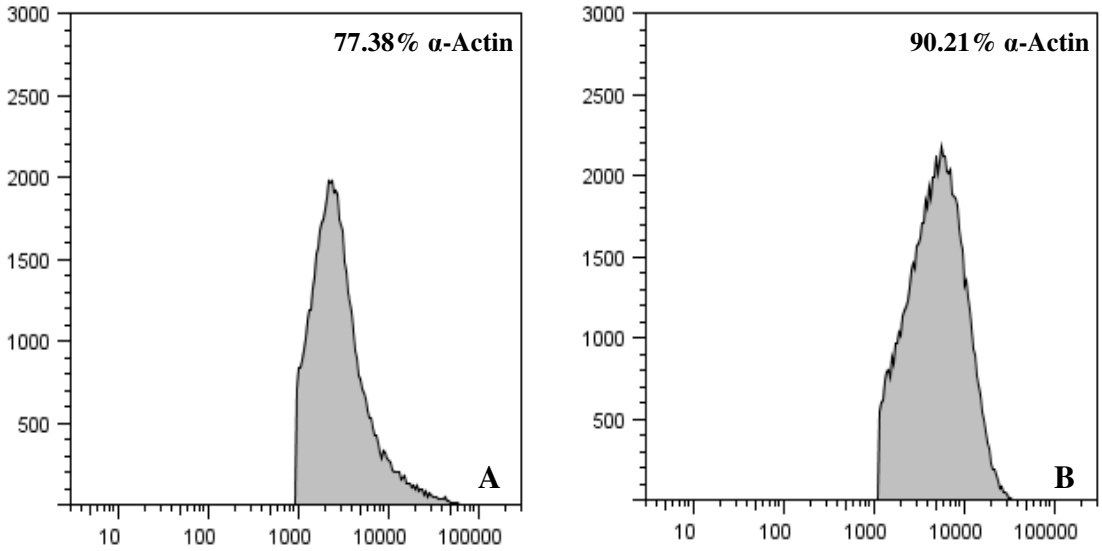

Figure 9: Representative flow cytometry plots of α-actin expression in control medium (containing TGF-β). Alpha smooth muscle actin expression in VICs was analyzed using Flow Cytometry at initial isolation (A), and 96 hours post-isolation on glass (B) cultured in control medium. In this representative experiment, freshly isolated VICs were 77.38% positive for alpha smooth muscle actin compared to VICs plated on glass for 96 hours, which were 90.21% positive for alpha actin. The y-axis of a histogram plot describes the number of cells per channel with each histogram containing 1024 channels (www.treestar.com).

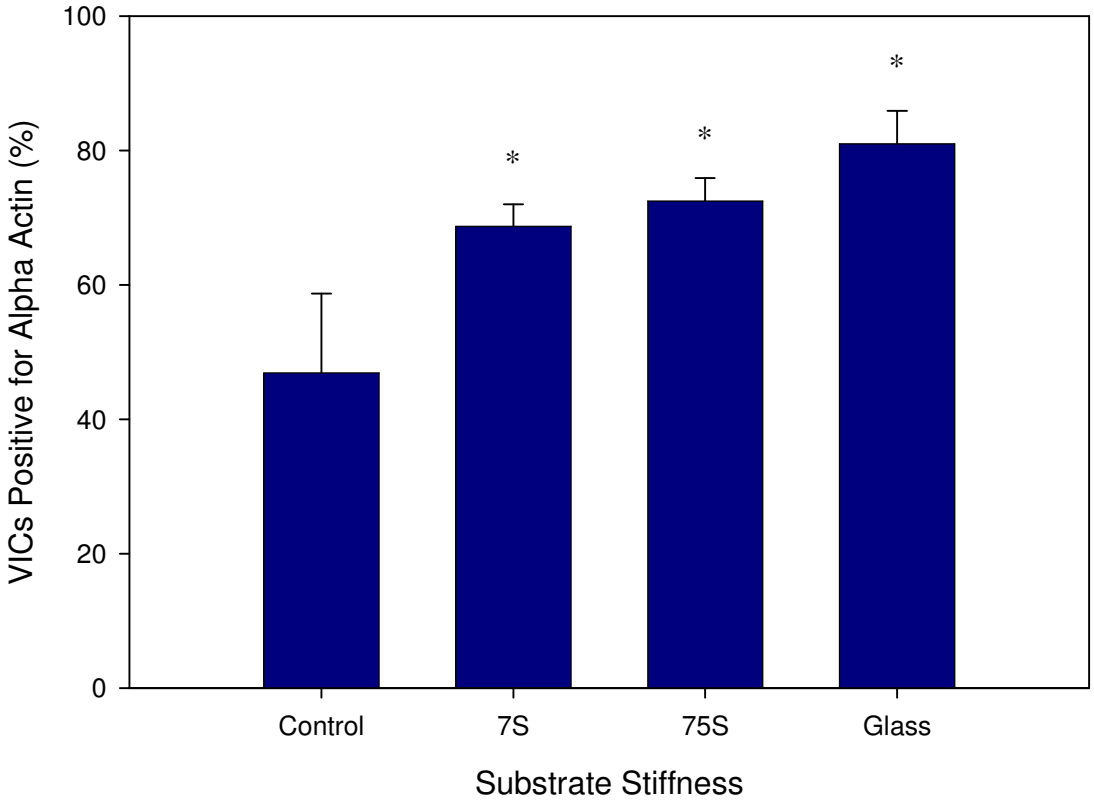

Figure 10: The effects of substrate stiffness on alpha actin expression of fresh isolates in control medium.

Alpha smooth muscle actin expression in VICs was analyzed using Flow Cytometry at initial isolation (Control), and 96 hours post-isolation on three varying substrate stiffnesses cultured in control medium: 7kPa (7S), 75kPa (75S), and glass. Alpha smooth muscle actin expression increased for all substrate groups in comparison to fresh isolates (n=4; * denotes p-value < 0.05).

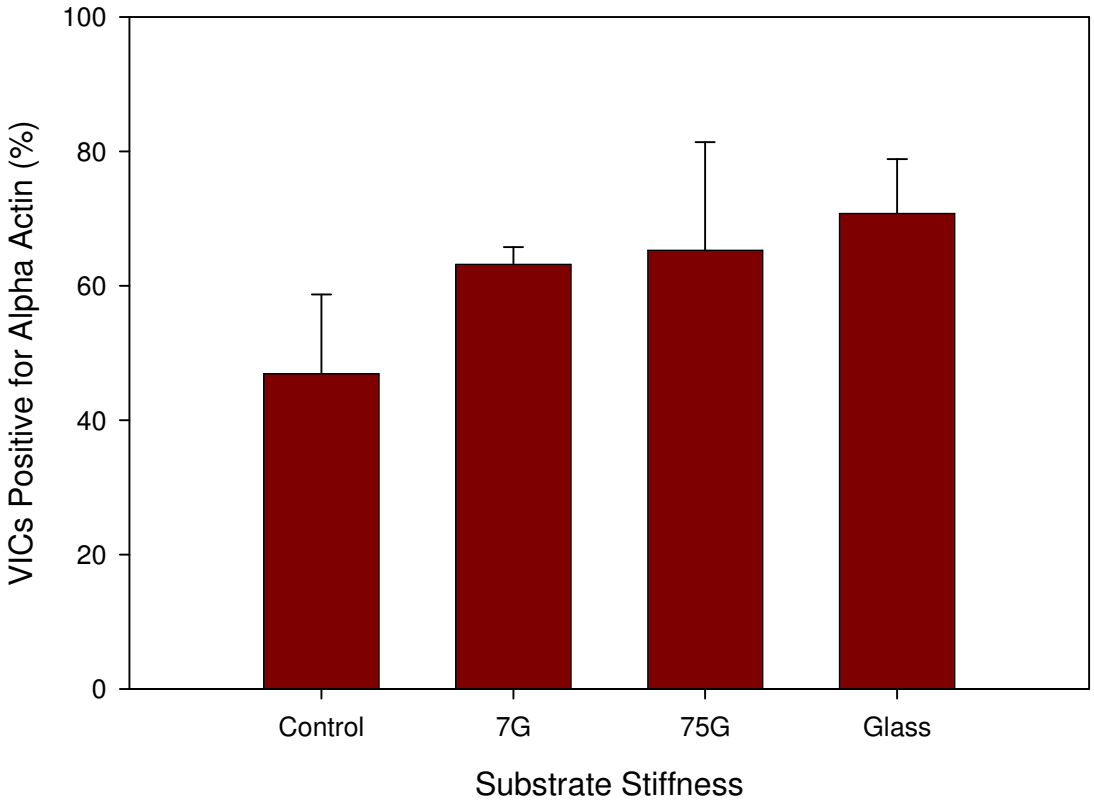

Figure 11: Effects of substrate stiffness on alpha actin expression of fresh isolates in the absence of TGF-β1.

Alpha smooth muscle actin expression in VICs was analyzed using Flow Cytometry at initial isolation (Control), and 96 hours post-isolation on three varying substrate stiffnesses cultured in TGF-β1 free medium: 7kPa (7S), 75kPa (75S), and glass. No statistical difference was observed between groups, although trends follow hypothesized results (n=4; $p > 0.1$).

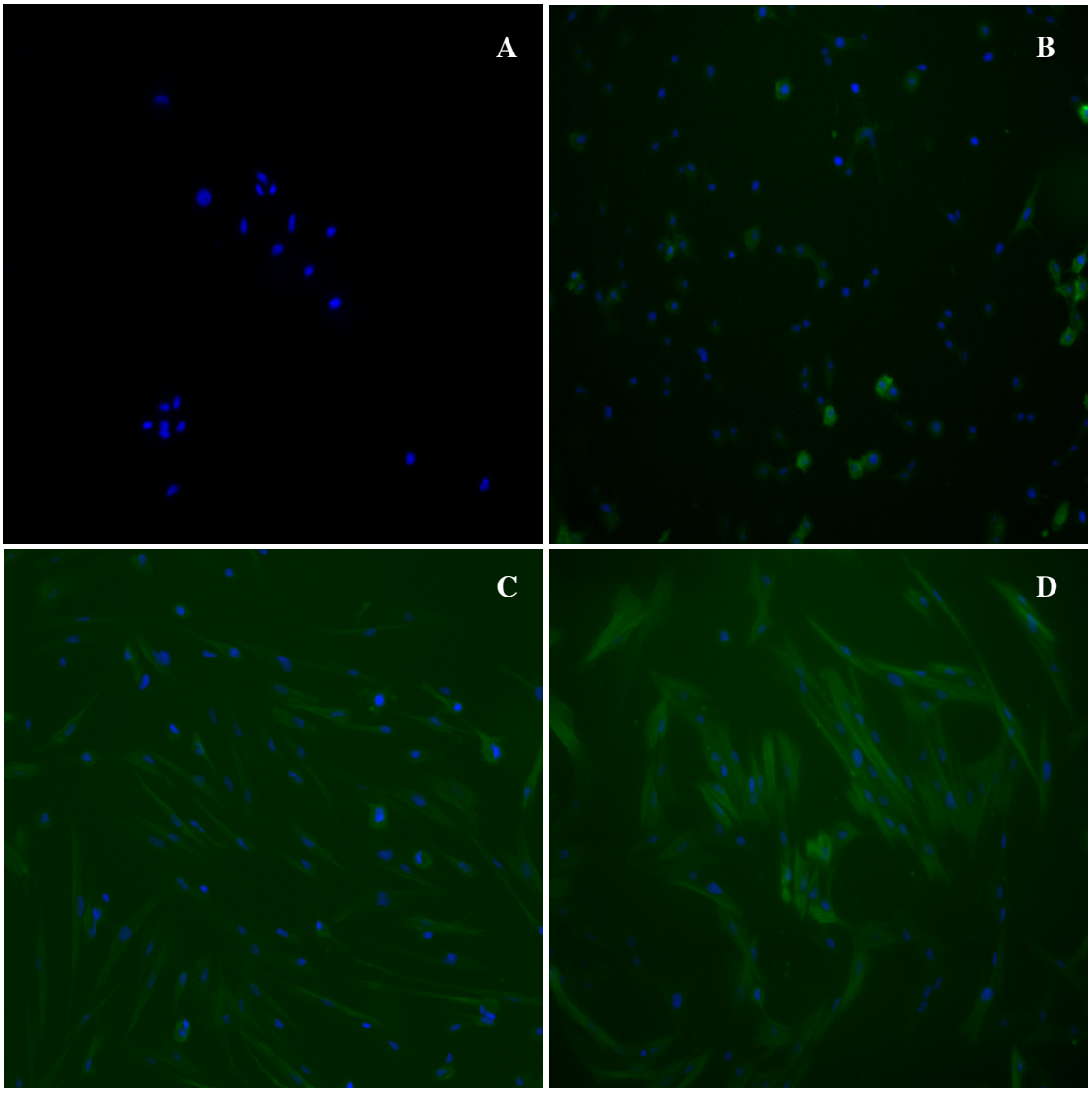

Figure 12: Immunocytochemistry of VIC Differentiation in Control Medium
VICs were cultured in control medium and analyzed for alpha smooth muscle actin in fresh isolates (A) and VICs plated for 96 hours on substrates of three varying stiffnesses: 7kPa(B), 75kPa (C), and glass (D). Alpha smooth muscle actin expression was minimal in VICs plated on 7kPa and 75kPa substrates. On VICs plated on glass, alpha actin expression was highly detectable with abundant stress fibers staining for alpha smooth muscle actin.

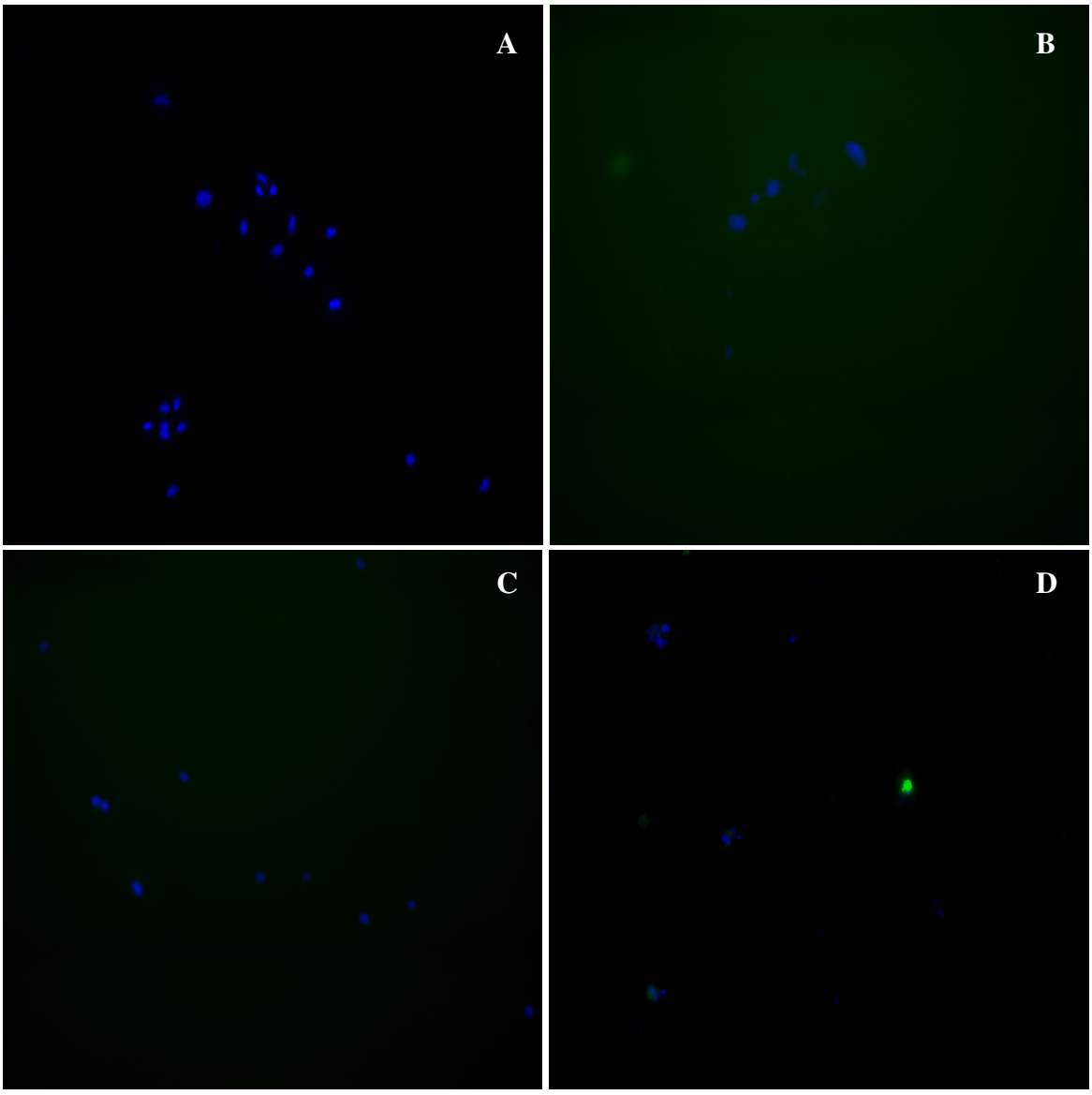

Figure 13: Immunocytochemistry of VIC Differentiation in TGF-β1 Free Medium
Cells were cultured in TGF-β1 free medium and analyzed for alpha smooth muscle actin expression. Fresh isolates (A) and VICs plated for 96 hours on substrates of three varying stiffnesses: 7kPa (B), 75kPa (C), and glass (D). Images showed little to no alpha actin expression in VICs grown on 7kPa and 75kPa substrates. On glass, VICs showed expression levels significantly less than the expression levels observed in VICs cultured on glass in control medium.

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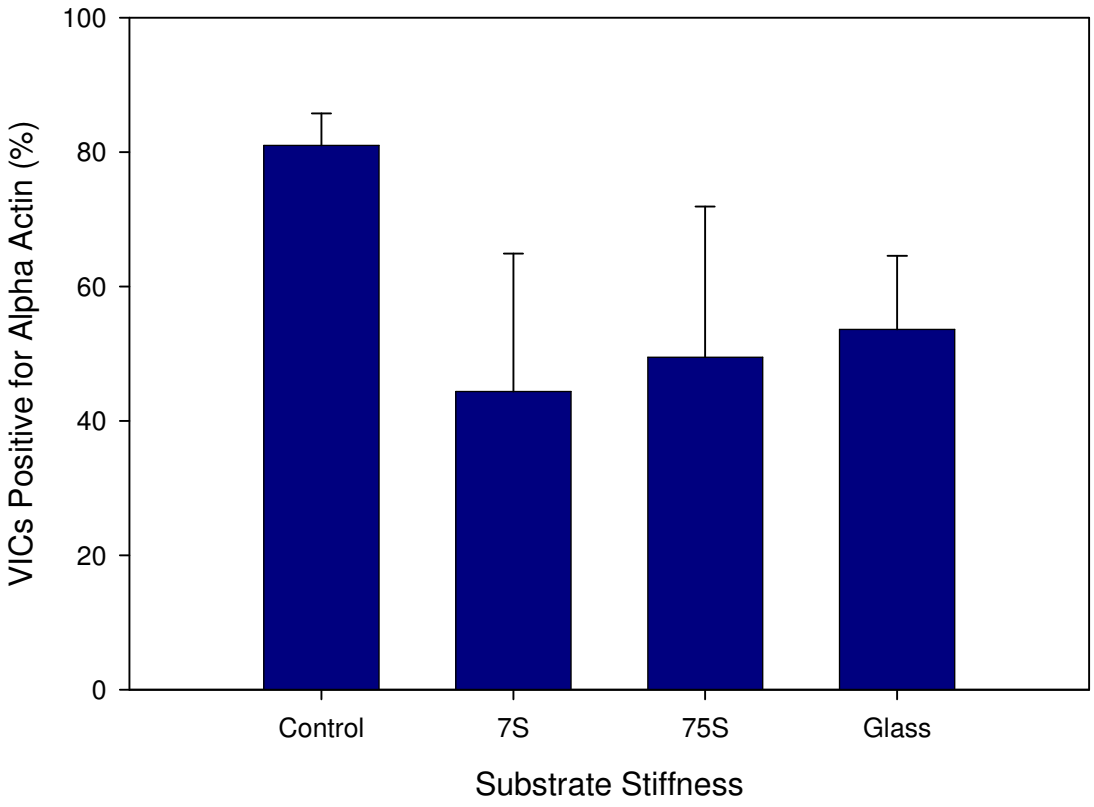

Figure 14: Dedifferentiation of VICs cultured in control medium

Alpha smooth muscle actin expression in VICs was induced by plating cells on plastic for one week in control medium (Control). After alpha actin induction, VICs were plated for 96 hours on three varying substrate stiffnesses cultured in control medium: 7kPa(7S), 75kPa (75S), and glass. A decrease in alpha smooth muscle actin was observed, although no statistical difference was calculated (n=3; p-value > 0.1).

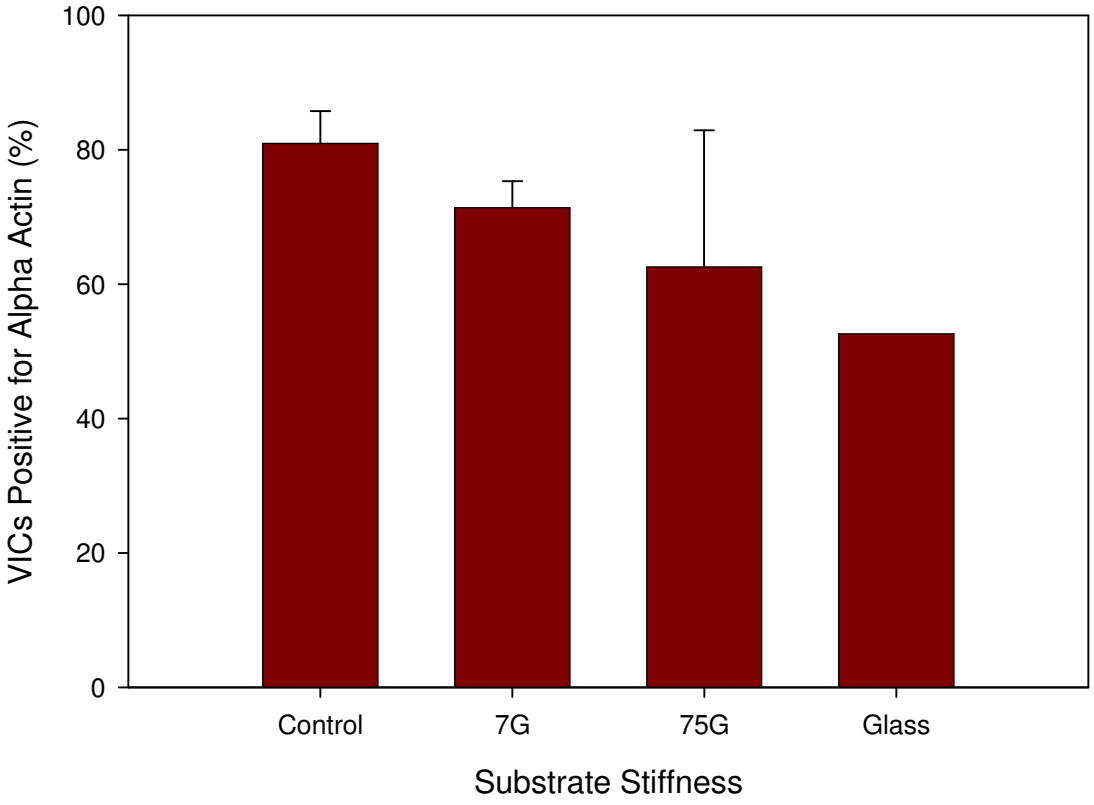

Figure 15: Dedifferentiation of VICs cultured in TGF-β1 free Medium

Alpha smooth muscle actin expression in VICs was induced by plating cells on plastic for one week in TGF-β1 free medium (Control). After alpha actin induction, VICs were plated for 96 hours on three varying substrate stiffnesses cultured in TGF-β1 free medium: 7kPa(7G), 75kPa (75G), and glass. There were no trends between experimental groups and no statistical significance observed (n=3; $p > 0.1$).

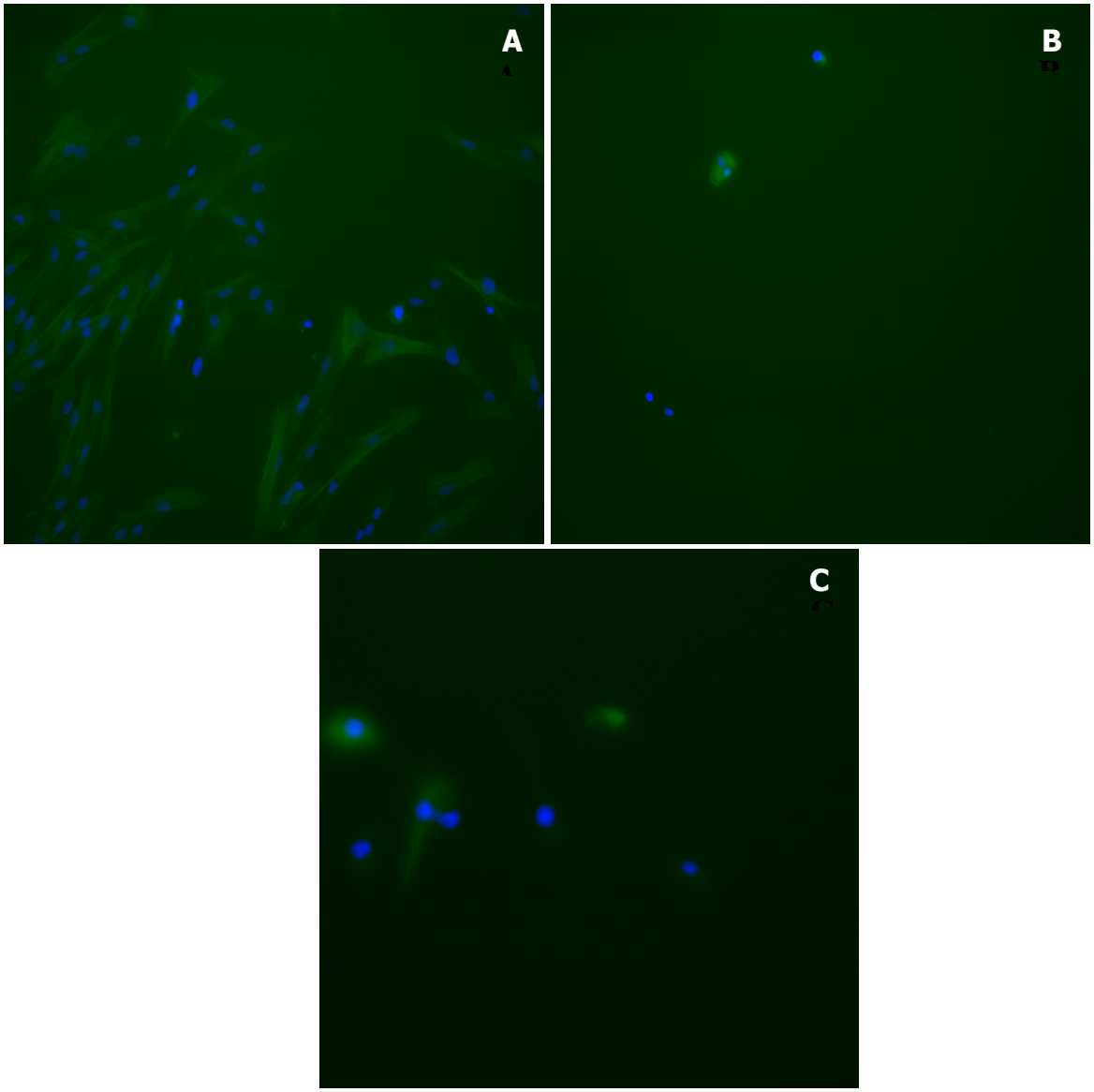

Figure 16 Immunocytochemistry of Dedifferentiation in Control Medium
Alpha smooth muscle actin expression was induced through plating VICs on tissue culture plating for a minimum of four days in the presence of control medium (A). After alpha actin induction, VICs were cultured in control medium for 96 hours on substrates of three varying stiffness': 7kPa (B), 75kPa (C), and glass. Images showed decreased levels of alpha actin expression in VICs grown on 7kPa and 75kPa substrates compared to fully differentiated VICs (A). On glass, VICs maintained similar expression levels to those that underwent alpha actin induction.

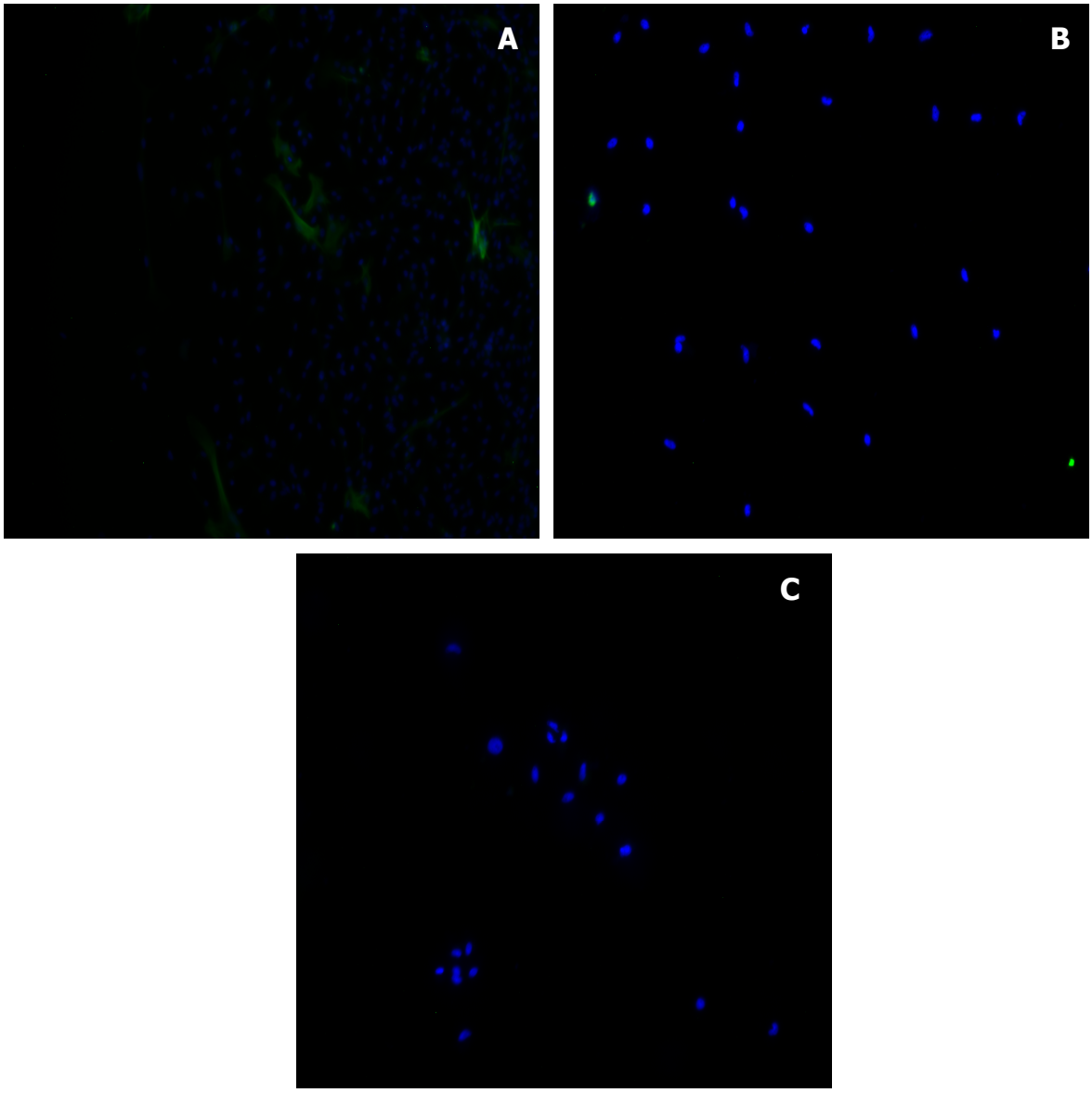

Figure 17 Immunocytochemistry of Dedifferentiation in TGF-ß1 Free Medium
Alpha smooth muscle actin expression was induced through plating VICs on tissue culture plating for a minimum of four days in the presence of TGF-β1 free medium. After alpha actin induction, VICs were cultured in TGF-β1 free medium for 96 hours on substrates of three varying stiffness': glass (A), 75kPa (B), 7kPa (C). Images showed no detectable alpha actin expression in VICs grown on 7kPa and 75kPa substrates. On glass, VICs maintained similar expression levels to those which underwent alpha actin induction.

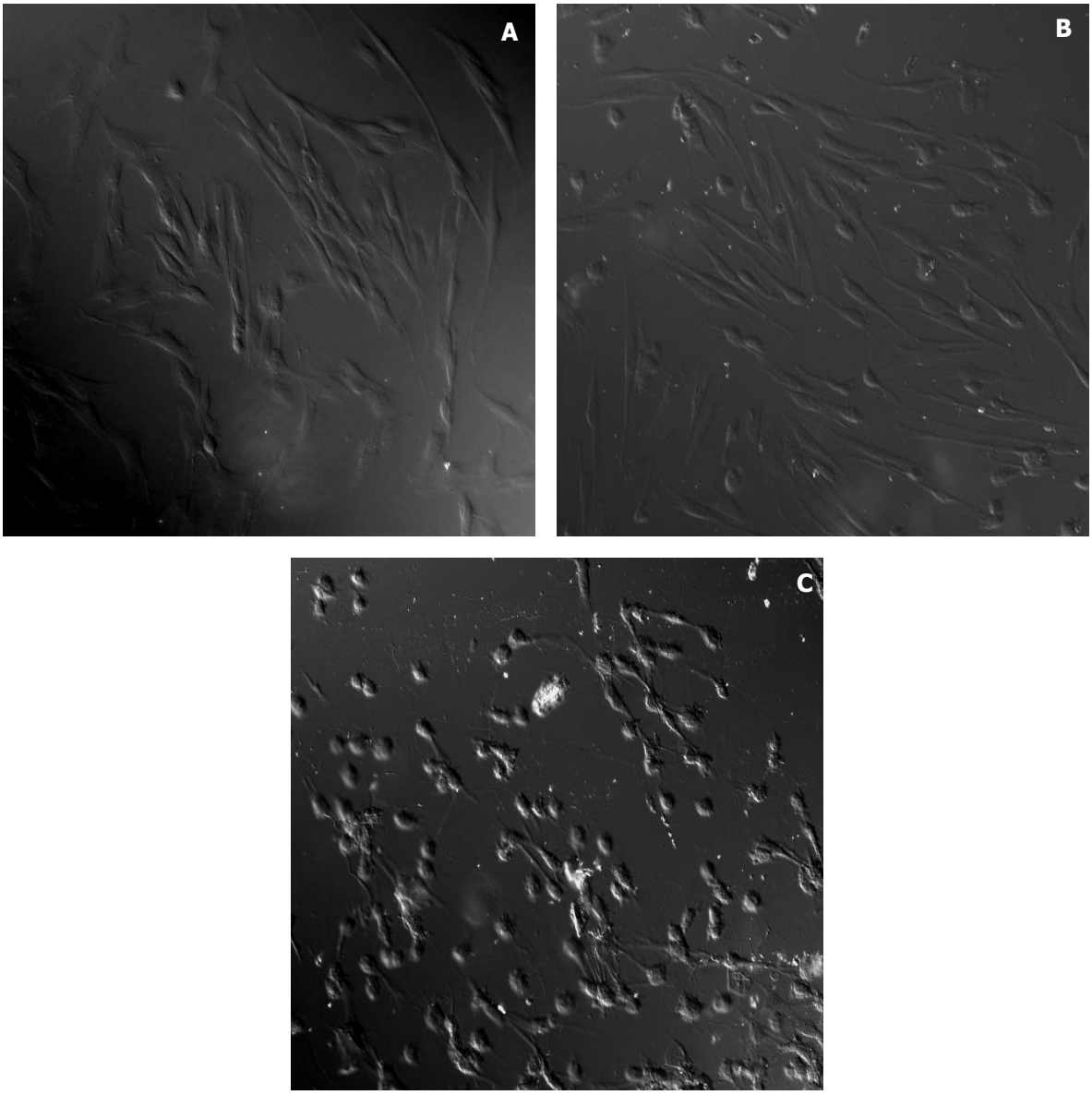

Figure 18 Dedifferentiation Cell Morphology of Cells Cultured in the Presence of Serum
Cell morphology was observed in VICs plated on the three substrates stiffnesses in the presence of serum: glass (A), 75kPa (B), and 7kPa (C). Results illustrated a change in cell morphology comparing glass, which is more polygonal in shape, to the 7kPa substrate which has a more rounded morphology, further supporting the possibility of dedifferentiation *in vitro*.

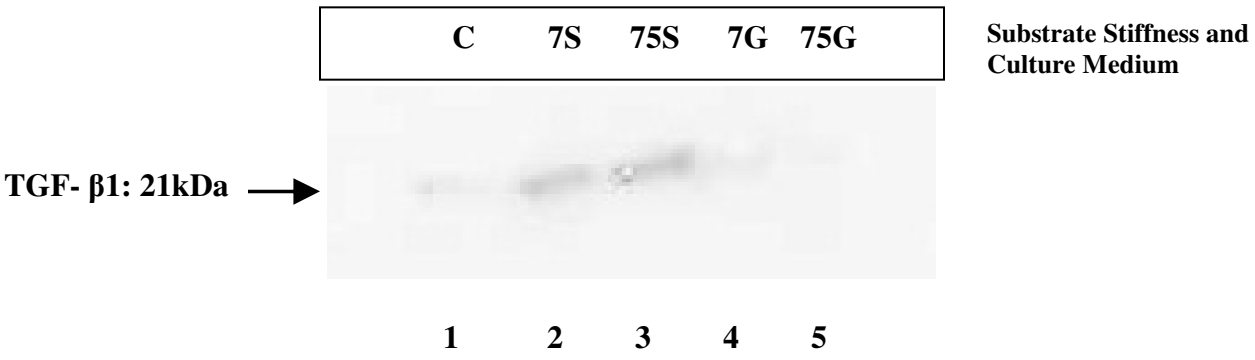

Figure 19 TGF-β1 Production in Various Media Types and Substrate Stiffnesses
VICs were plated in either control medium (S) or growth factor free medium (G) on substrates of varying stiffness (7kPa and 75kPa). Immunoblots were probed for TGF- β1 and a 21kDa band was detected for all experimental groups. In the presence of control medium, TGF- β1 production was greater in comparison to growth factor free medium and fresh isolates (C). Comparing the varying stiffness among similar medium types, TGF- β1 production also increased slightly between the 7kPa and the 75kPa substrate stiffness.

Discussion

Differentiation

The aim of this study was to determine whether substrate stiffness modulates the amount of alpha smooth muscle actin expression in valvular interstitial cells. The concept that a mechanical stimulus can initiate the myofibroblastic differentiation pathway has been proposed and demonstrated by (Gabbiani 2003) and several other researchers in other cell systems (MacKenna, Summerour et al. 2000; O'Callaghan and Williams 2000; Durbin and Gotlieb 2002; Engler, Griffin et al. 2004). The mechanical stimulus used here was substrate stiffness meant to model changes in tissue stiffness in the pathologic state. Based on the concept that mechanical stimulation would initiate the pathway, one would expect little or no alpha actin expression on substrates with normal physiological stiffness levels (MacKenna, Summerour et al. 2000; Engler, Griffin et al. 2004), such as the 7kPa substrate used in these particular experiments. This would correspond to the normal situation *in vivo* where the number of myofibroblasts is quite low. The 75kPa substrate used mimicked a minimally pathological state, and one would expect to see a slight increase in alpha smooth muscle actin expression in cells plated on these substrates. Glass, the final substrate used in this project, has a stiffness level in the range of gigapascals and is hypothesized to mimic pathological environments *in vivo* (Engler, Griffin *et al.* 2004) as well as the *in vitro* conditions that have been shown to induce alpha smooth muscle actin expression (Yperman, De Visscher et al. 2004).

In the freshly isolated porcine mitral valvular interstitial cells used here, the average alpha actin expression level was approximately 50% with significant variability between individual preparations. According to a study performed to characterize various valve types for healthy human heart valves, this value is significantly higher than that reported by (Rabkin-Aikawa, Farber et al. 2004). However, a second study by (Durbin and Gotlieb 2002) indicated that up to 50% of the cellular population of some normal valves may be activated, alpha smooth muscle actin expressing valvular interstitial cells, in agreement with this thesis.

The hearts used as a source of cells for this study came from a local abattoir and thus were subject to both genetic and physiologic variability. No attempt was made to control for strain, age, gender, or weight, all of which may have contributed to the significant variability among cell preparations. Additionally, initial cell preparations were not monitored for purity using a fibroblast marker such as vimentin due to difficulties in signal detection possibly due to significant autofluorescence of the cells at the wavelength needed to detect vimentin, 405nm.

While smooth muscle cells are unlikely to be significant contaminants of these preparations, the potential for variability of cell types in the initial preparations or transdifferentiation of other cells types, such as valvular endocardial cells to a phenotype that would express alpha smooth muscle actin cannot be ruled out.

Using these alpha actin expression levels obtained from fresh isolates as baseline values, flow cytometry was used to compare the alpha actin expression levels of mitral valvular interstitial cells cultured under various conditions. VICs were cultured for 96 hours on three stiffnesses of substrate in the presence of two different media. The two media used were control medium, which contained 10% fetal bovine serum (and presumably TGF-β), and TGF-β-free medium, which contained no exogenous growth factors except basic fibroblast growth factor (bFGF) added as a supplement. bFGF was added to the medium at 2ng/ml as a mitogen because VIC replication slowed significantly in the absence serum. The main reason for using two different media was to observe whether autocrine TGF- β1 production is upregulated when cells are plated on substrates which would hypothetically initiate the differentiation pathway and whether this level of TGF-β1 production would be sufficient to allow differentiation to the myofibroblast phenotype (Gabbiani 2003).

VICs cultured in control medium showed an increase in alpha actin expression for all substrate stiffness' with the highest level of alpha actin expression on glass (Figure 10). An increase on the 7kPa substrate was unexpected since this was meant to mimic the normal tissue stiffness. However the actual stiffness level within the heart valve is unknown. As myotubes have a very narrow range of stiffness for optimal differentiation (Engler, Griffin et al. 2004), valvular interstitial cells may experience stiffness levels *in vivo* slightly below the 7kPa substrate used for experimentation. The VICs may be sensitive to any stiffness level outside of their physiological range and as a result would upregulate alpha actin. The results for cells grown on the 75kPa and glass substrates were expected with substantial increase in alpha actin expression levels in both groups compared to fresh isolates. Immunocytochemistry of VICs cultured in control medium for the duration and substrates mentioned previously, illustrated that the 7kPa and 75kPa substrates expressed minimal of increased amounts of alpha actin, cells grown on glass, the stiffest substrate, had the most expression with prominent stress fibers characteristic of activated VICs, staining positive for alpha actin (Figure 12). While the results from flow cytometry and immunocytochemistry were not precisely the same, both indicated clearly that substrate stiffness does have an effect on VIC activation as demonstrated by increased alpha smooth muscle actin expression.

VICs cultured in TGF-β1 free medium (Figure 11) exhibited no statistically significant difference in alpha actin expression between experimental groups compared to fresh isolates. There are a number of reasons this may have occurred. The first and most important reason this may have occurred is that in the absence of TGF-β1, autocrine TGF-β1 levels may not have been adequate to induce alpha actin expression. TGF-β1 has been shown to play a key role in myofibroblast differentiation (Zhou, Ono et al. 1997; Dooley, Delvoux et al. 2000; Vaughan, Howard et al. 2000; Gabbiani 2003; Walker, Masters et al. 2004; Kopp, Preis et al. 2005) as well as in other cell types (Desmouliere, Geinoz et al. 1993; Arora and McCulloch 1999; Jester, Huang et al. 1999). Most *in vitro* experiments similar to the ones performed in this project have been completed in the presence of serum, which contains numerous growth factors, including TGF-β1. *In vivo*, TGF-β1 comes from various sources within the body. The combination of autocrine and paracrine TGF-β1 signaling provides sufficient levels to maintain the differentiation pathway (Gabbiani 2003). While autocrine production may be sufficient to maintain the differentiation of myofibroblasts once fully activated *in vivo*, paracrine signaling may be required to initiate the differentiation program. Additionally, because the number of cells was deliberately kept at a subconfluent level, the TGF-β levels produced in the culture may not have reached the level necessary to complete the differentiation to the myofibroblast phenotype. *In vitro*, fetal bovine serum may play the role of paracrine production *in vivo.*

Results of immunoblots for TGF-β1 confirmed that intracellular levels of TGF-β1 were considerably higher in VICs cultured in the presence of serum versus TGF-β1-free medium compared to fresh isolates (Figure 19). This result was independent of the substrate stiffness they were plated on. This may suggest that *in vitro* serum contains cofactors that promote the autocrine production of TGF-β1. Since the only growth factor contained in the TGF-β-free medium was bFGF, this medium may have been insufficient to support production of TGF-β1 by these cells. This may also suggest that paracrine production of TGF-β1 is required to continue the differentiation of VICs *in vivo*. The high levels of intracellular TGF-β1 in the presence of serum suggest that paracrine production may promote the autocrine production of TGF-β1 *in vivo.*

Dedifferentiation

As stated above, the goal of these studies was to determine whether substrate stiffness modulates alpha actin production of valvular interstitial cells *in vitro.* The activation of this particular VIC cell type has not been studied in great detail, yet much is known regarding the differentiation of myofibroblasts in other tissues. Results from the previous section confirm that

in the presence of serum *in vitro*, alpha actin expression increases on all three substrates and that serum plays a role in the autocrine production of TGF-β1 *in vitro*.

If VIC dedifferentiation to the initial fibroblastic phenotype is possible *in vivo*, VICs should have the potential to return to the quiescent inactive state *in vitro* if cultured in appropriate conditions. To explore this possibility, flow cytometry and immunocytochemistry were utilized to test for a decrease in alpha actin expression, the defining marker for the level of VIC activation under varying culture conditions. Alpha actin expression first had to be induced in a significant number of cells in order to observe any potential decrease in expression levels. This was achieved through plating VICs on tissue culture plastic for a minimum of four days in the appropriate culture medium. Following alpha actin induction, VICs were re-plated in the appropriate medium for 96 hours on the same substrates used for differentiation experiments.

Immunocytochemistry results from both experimental groups, TGF-β1-free medium and control medium, displayed a decrease in alpha actin expression on the 7kPa and 75kPa substrates in contrast to the fully differentiated VICs (Figure 16 and Figure 17). While both groups displayed a decrease in alpha actin expression, cultured VICs in TGF-β1- free medium actually showed no discernible alpha actin expression on these two substrates. Glass substrates for each group maintained similar levels of alpha actin expression as the fully differentiated VICs. These results supported the hypothesis that substrate stiffness modulates alpha actin expression by showing a decrease on substrates with stiffness levels closer to physiological conditions *in vivo*. In the absence of exogenous TGF-β1, alpha actin expression was in fact undetectable on substrates mimicking stiffness levels close to those found *in vivo*.

Unfortunately, flow data showed no statistically significant difference under either experimental culture condition (Figure 14 and Figure 15). However, in the presence of serum, expected trends were observed; although the variability within groups was too high to detect any statistical difference. Immunoblots for TGF-β1 were also run and no detectable levels were found in any experimental group except for the control group of fully differentiated VICs and VICs cultured on glass in the presence of serum (data not shown). This is consistent with the data shown in Figure 19, which was performed under the differentiation regime and warrants further study. Perhaps it is not the substrate stiffness per se, but a change in substrate stiffness that regulates the differentiation pathway.

The Model

Prior to the start of this project, a model was created to aid in the experimental design and interpretation of collected data (Figure 20). The model describes the differentiation and

dedifferentiation of porcine mitral valvular interstitial cells *in vitro*. It begins with an inactive VIC in its quiescent fibroblast-like state with minimal alpha actin expression (left side of diagram). This quiescent VIC is then plated on a stiff substrate, which initiates the "activation" or differentiation of the VIC. This activation is indicated through the increase of alpha actin expression and intracellular TGF-β1 levels (top portion of diagram). The combination of the stiff substrate and TGF-β1 results in a fully "activated" or differentiated VIC (right side of diagram). Comparing hypothesized results for the differentiation portion of the model, it was shown in this study that stiff substrates do in fact cause an increase in alpha actin expression. Also in the presence of serum, intracellular TGF-β1 levels were significantly higher in contrast to VICs exposed to growth factor-free culture conditions.

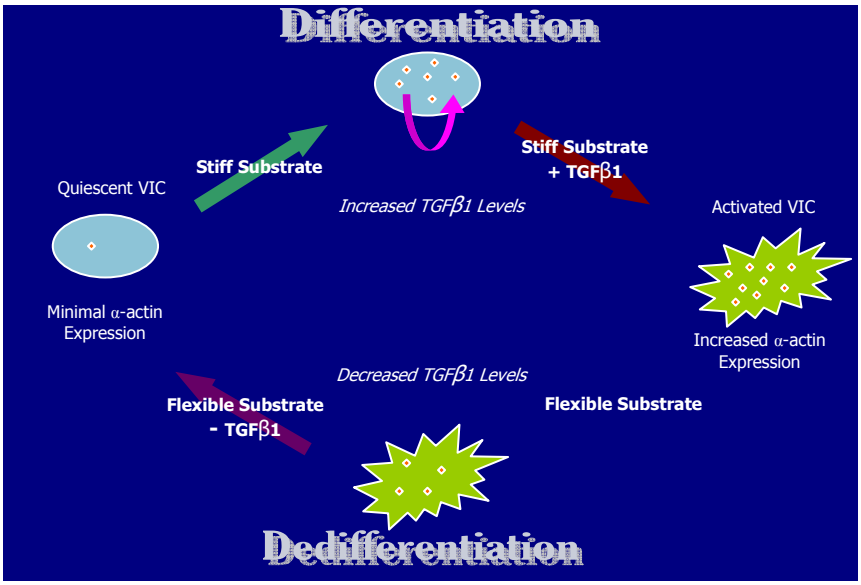

Figure 20 The proposed model for VIC differentiation and dedifferentiation
This model illustrates the hypothesized differentiation and dedifferentiation of valvular interstitial cells *in vitro*. VICs become "activated" or differentiated indicated by an increase in alpha actin expression through the combination of stiff substrates and TGF-β1. The dedifferentiation, shown by a decrease in alpha actin expression, occurs by grouping substrates of physiologic stiffness with a decrease in TGF-β1 levels.

The dedifferentiation of VICs proposed in this model occurs through plating the fully differentiated, "activated" VIC on substrates which mimic stiffness levels *in vivo.* This results in a decrease of alpha smooth muscle actin expression as well as intracellular TGF-β1 levels, indicative of the possible dedifferentiation of the "activated" VICs. The combination of decreased TGF-β1 levels and physiological substrate stiffness results in a decrease of alpha actin expression and therefore the return of the VIC to its "inactive" quiescent state. Comparing this model to the results gathered during this study demonstrates that alpha actin expression does in fact decrease when plated on softer substrates in comparison to glass, indicating the possibility

for dedifferentiation. Cell morphology in the presence of serum further supports the indication of VIC dedifferentiation *in vitro*, though further research must be completed before conclusions can be drawn regarding this possibility (Figure 18). Unfortunately, TGF-β1 levels were undetectable in growth factor-free and serum culture conditions except when cultured on glass in the presence of fetal bovine serum. Further studies need to be completed before conclusions can be drawn regarding the levels of intracellular TGF-β1 in the dedifferentiation of VICs *in vitro*.

If, as appears to be the case, the cells can be induced to dedifferentiate, the possibility of using a patients own cells to populate artificial valve scaffolds becomes real. This would avoid several complications associated with current treatments, including immune rejection. Whether the dedifferentiation can be induced *in vivo,* by simply providing a scaffold with mechanical properties which mimics normal tissue or whether the cells would need to be harvested in a valve reconstruction and dedifferentiated *in vitro* requires further study. We cannot rule out the possibility that apparent decreases in alpha smooth muscle actin expression after plating fully differentiated VICs on "soft" substrates is a result of myofibroblast apoptosis and proliferation of inactive VICs. However, there was no evidence of significant changes in cell morphology consistent with apoptosis and no increase in cell debris. Perhaps apoptosis could be tested in future experiments using an immunohistochemical analysis of apoptotic marker proteins. Additional studies will be needed to further explore this exciting valvular replacement possibility.

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